IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

APPLICANT: Rosenblum *et al.*	§	**ART UNIT:**
	§	**1642**
FILED: May 26, 1999	§	
	§	**EXAMINER:**
SERIAL NO.: 09/320,156	§	**Canella, K.**
	§	
FOR: Immunotoxins Directed Against c-erbB-2 (HER-2/Neu) Related Antigens	§	**DOCKET:**
	§	**D5425CIP2**

The Honorable Commissioner of Patents
Washington, DC 20231

ATTENTION: Board of Patent Appeals and Interferences

TRANSMITTAL OF APPEAL BRIEF

Dear Sir:

Enclosed please find three (3) originals of the Appeal Brief for the above-referenced patent application.

This application was submitted on behalf of a small entity. A Verified Statement of Small Entity Status was included with the patent application. Please debit the $160 fee for filing a brief in support of an appeal under 35 U.S.C. 1.17(c) or any additional fees due from Deposit Account No. 07-1185 on which account the undersigned is allowed to draw.

Respectfully submitted,

Date: Jan 13, 2003

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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

APPLICANT: Rosenblum *et al.*	§	**ART UNIT:** 1642
FILED: May 26, 1999	§	
SERIAL NO.: 09/320,156	§	**EXAMINER:** Canella, K.
FOR: Immunotoxins Directed Against c-erbB-2 (HER-2/Neu) Related Antigens	§	**DOCKET:** D5425CIP2

The Honorable Commissioner of Patents
BOX AF
Washington, DC 20231

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CERTIFICATE OF MAILING UNDER 37 C.F.R. 1.8

Dear Sir:

I hereby certify under 37 CFR 1.8 that the following correspondence is being deposited with the United States Postal Service as first class mail with sufficient postage on the date indicated below and is addressed to The Honorable Commissioner for Patents, Washington DC 20231.

(1) Appeal Brief;
(2) Return Postcard.

Please return the enclosed postcard acknowledging receipt of this correspondence.

Respectfully submitted,

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OF THE PATENT OFFICE, PLACED HEREON, ACKNOWLEDGES RECEIPT OF:

Applicant: Rosenblum, et al. Attorney: Benjamin Aaron Adler

Serial No: 09/320,156 Docket No.: 5425C1P2 Date: 1/13/03

[ ] Provisional Patent Application w/coversheet
[ ] Sequence Listing w/ Compliance Letter + CRF
[ ] Application for Patent including [ ] PCT
_ pages spec. _total claims
[ ] Request for filing [ ] Continuation
[ ] Divisional
[ ] Request for Continued Examination
[ ] Drawings:_ sheets [ ] informal [ ] formal
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OF THE PATENT OFFICE, PLACED HEREON, ACKNOWLEDGES RECEIPT OF:

Applicant: Rosenblum, et al. Attorney: Benjamin Aaron Adler

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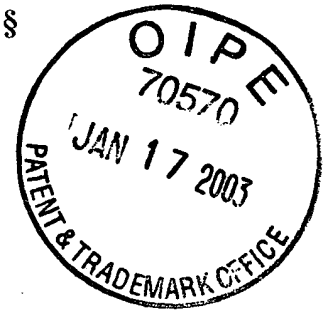

The Honorable Commissioner of Patents
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ATTENTION: BOARD OF PATENT APPEALS AND INTERFERENCES

APPELLANT'S BRIEF

This Brief is in furtherance of the Notice of Appeal filed in this case on November 22, 2002. The fees required under 37 C.F.R. §1.17(f) and any other required fees are dealt with in the accompanying TRANSMITTAL OF APPEAL BRIEF.

In accordance with 37 C.F.R. §1.192(a), this Brief is submitted in triplicate.

INDEX OF SUBJECT MATTER

I. REAL PARTY IN INTEREST

The real party in interest is the Research Development Foundation, the Assignee, as evidenced by an Assignment recorded in the Patent and Trademark Office at Reel 012024, Frame 0741 on July 24, 2001.

II. STATUS OF THE CLAIMS

Originally claims 1-21 were filed with this Application. Claims 1-14 and 20-21 were withdrawn from consideration. Claims 15-19 were amended. The pending claims 15-19 are being appealed, of which claim 15 is an independent claim.

III. STATUS OF AMENDMENTS

Subsequent to the final rejection mailed May 23, 2002, Applicants submitted a Response After Final which amended claims

16-18 and cancelled claims 15 and 19. In an Advisory Action mailed November 19, 2002, the amendments were not entered into the record. All pending claims are shown in Appendix A.

IV. STATEMENT OF RELATED APPEALS AND INTERFERENCES

To Appellant's knowledge, there are no pending related appeals or interferences that will directly affect or be directly affected by the present appeal.

V. SUMMARY OF THE INVENTION

The present invention is directed to a composition comprising a conjugate of a cellular targeting moiety, *e.g.*, an antigen binding region, exhibiting binding specificity for the c-erbB-2 protein and a cell growth modulator, *e.g.*, a toxin or growth suppressing reagent (see the Specification at page 3, lines 18-21. This composition acts as an immunotoxin to specifically target a cell growth modulator to tumor cells overexpressing the c-erbB-2 protein

(page 4, lines 1-2). In one embodiment, the composition may be a fusion construct between a targeting moiety with binding affinity for c-erbB-2 protein and a cytotoxic moiety (page 4, lines 16-19). In a preferred embodiment, the conjugate is a fusion protein between a single chain antibody and tumor necrosis factor which is preferable produced by recombinantly fusing a gene encoding a single chain antibody to a gene encoding tumor necrosis factor (page 5, lines 19-21 and page 6, lines 1-2). One possible single chain antibody is scFv-23 (page 6, line 2). The present invention is useful for treating target neoplastic cells that overexpress the c-erbB-2 protein on the cell surface (page 4, lines 10-15).

VI. ISSUES

A. 35 U.S.C. §103(a)

Whether claims 15-19 are patentable under 35 U.S.C. §103(a).

VII. GROUPING OF CLAIMS

The rejected claims do not stand or fall together. Applicants consider that claims 16-18 are separately patentable from claims 15 and 19. Applicants submit that a composition comprising a conjugate which is a fusion protein between a single chain antibody and tumor necrosis factor is patentably distinct from such conjugates produced by other means.

VIII. ARGUMENTS

The Rejection Under 35 U.S.C. §103(a)

In the Advisory Action mailed November 19, 2002, the Examiner maintained the rejection of claims 15-19 under 35 U.S.C. §103(a) for substantially the same reasons found in the Final Office Action mailed May 23, 2002, as being unpatentable over **Wels** *et al.* (U.S. Pat. No. 5,571,894) in view of **Hoogenboom** *et al.* (*Biochimica et Biophysica Acta* 1096(4): 345-354 (1991), Abstract) and **Hudziak** *et al.* (*Mol. Cell. Biol.* 9(3): 1165-1172 (1989)). Applicants vigorously traverse this rejection.

In the Advisory Action mailed November 19, 2002, the Examiner maintained the rejection of claims 15-19 under 35 U.S.C. §103(a) for reasons of record. The Examiner asserted:

> **Wels** teaches an anti-erbB2 antibody which is a single chain antibody, fused to a plant bacterial toxin. **Hoogenboom** teaches a single chain antibody-TNF fusion protein. Applicant argues the same point against the combination of **Wels** and **Hoogenboom** and **Hudziak** as above. It is not found persuasive for the reasons set forth above, namely that **Wels** teaches that a single chain antibody can be fused to a bacterial toxin and retain the binding activity of the antibody as well as the toxic activity associated with anti-ribosomal plant toxins. **Hoogenboom** teaches that TNF can be fused to a single chain antibody with retention of the antibodies [sic] binding activity, and the retention of TNF activity, and the resulting immunotoxin is useful as a toxic agent against myeloma cells. **Hudziak** teaches the efficacy of providing TNF simultaneous[ly] to anti-erbB2 in the treatment of breast cancer cells. Thus, one of skill in the art would be motivated to make an immunotoxin to target breast cancer cells by the fusion of an anti-erbB2 antibody to TNF from the teachings of **Wels** and **Hudziak**. One of skill in the art would have reasonable expectation of success from the teachings of **Hoogenboom** on the retention of binding and TNF activity in the anti-myeloma TNF immunotoxin.

The Examiner maintains that one of skill in the art would be motivated to make an immunotoxin to target breast cancer cells by fusing an anti-erbB2 antibody to TNF from the teachings of **Wels** and **Hudziak**, and would have a reasonable expectation of success from the teachings of **Hoogenboom** of retention of antibody binding and TNF activity. Applicants respectfully disagree.

Applicants respectfully submit that given the structural specificity and precision in the process of protein folding that defines each protein's activity, one skilled in the art would not be motivated to combine the teachings of **Wels** or **Hoogenboom** and **Hudziak** with any expectation of success of obtaining the particular composition in the present claims while retaining both antibody binding and cytotoxic activity.

The biological activity of a protein depends on the folding of its amino acid chain(s) into a highly organized, precise three-dimensional structure under physiological conditions (*The Encyclopedia of Molecular Biology*, Sir John Kendrew Ed., Blackwell Science Ltd., London, p. 884 (1994)). In **Hudziak**, the separate administration of un-conjugated monoclonal anti-erbB2 antibody and

TNF sensitized breast cancer cells to TNF. The instant invention improves on **Hudziak** by administering TNF concurrently with the sensitizing antibody. However, one skilled in the art at the time of the invention could not determine from the teachings of **Wels** or **Hoogenboom** and **Hudziak** whether a fusion product between TNF and a single-chain anti-erbB2 antibody would retain antibody binding and TNF toxicity properties.

It is likely that the fusion process would result in the disruption of the specific processes of protein folding necessary for the separate proteins to function properly. For example, **Friedman** *et al.* (*Cancer Research* 53: 334-339 (1993)) report that investigators in the art have experienced a reduction in binding efficiency of 3- to 10- fold in recombinant antibody proteins compared to the corresponding non-recombinant IgG antibodies. In **Friedman**, a fusion protein formed between a single-chain sFv antibody and a *Pseudomonas* exotoxin fragment resulted in the production of a mixture of monomeric and aggregate proteins. The monomer was 5-fold less effective than the native IgG in binding to the target antigen, whereas the aggregate form was unable to bind at all. The aggregates mainly resulted from monomers that were misfolded

during production, forming inappropriate disulfide bonds, thus hindering the correct three-dimensional display of antigen-combining sites in the antibody. Further experiments were therefore necessary to increase the antigen-binding activity of the recombinant immunotoxin molecule. In further example, in **Chaudhary** *et al.* (*Nature* 339: 394-397 (1989)), a fusion protein made between a single-chain antibody and a modified form of *Pseudomonas* exotoxin displayed a 3-fold decrease in binding to the target antigen over the un-fused antibody.

Given the difficulty experienced in the art in producing active immunotoxins as fusion proteins, it is therefore likely that time-consuming, non-routine experimentation, beyond that which is obvious to one skilled in the art, would be required to determine if the anti-erbB2-TNF fusion protein would exhibit the same or better cytotoxic effects as the separate administration of each component. Applicants therefore respectfully submit that claims 15-19 are not obvious under 35 U.S.C. §103(a) over **Wels** in view of **Hudziak** and **Hoogenboom**. Accordingly, Applicants respectfully request that the rejections of claims 15-19 under 35 U.S.C. §103(a) be withdrawn.

For the reasons given above, Applicants respectfully urge that the decision of the Examiner should be reversed, and that claims 15-19 be allowed.

Respectfully submitted,

Date: Jan 13, 2003

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APPENDIX A

CLAIMS ON APPEAL

15. A composition comprising a conjugate of tumor necrosis factor to an antibody exhibiting binding specificity for an extracellular epitope of c-erbB-2 protein.

16. The composition of claim 15, wherein said conjugate is a fusion protein between said single chain antibody and tumor necrosis factor.

17. The composition of claim 15, wherein said conjugate is recombinantly produced by fusing a gene encoding said single chain antibody to a gene encoding tumor necrosis factor.

18. The composition of claim 15, wherein said single chain antibody is scFv-23.

19. A pharmaceutical composition, comprising the composition of claim 15 and a pharmaceutically acceptable vehicle.

APPENDIX B

L8 ANSWER 9 OF 30 MEDLINE
ACCESSION NUMBER: 91291906 MEDLINE
DOCUMENT NUMBER: 91291906
TITLE: Targeting of **tumor necrosis factor** to tumor cells: secretion by myeloma cells of a genetically engineered **antibody-tumor necrosis factor** hybrid molecule.
AUTHOR: Hoogenboom H R; Raus J C; Volckaert G
CORPORATE SOURCE: Dr. L. Willems-Instituut en Departement WNIF, Limburgs Universitair Centrum, Diepenbeek, Belgium.
SOURCE: BIOCHIMICA ET BIOPHYSICA ACTA, **(1991 Jun 5)** 1096 (4) 345-54.
Journal code: A0W. ISSN: 0006-3002.
PUB. COUNTRY: Netherlands
Journal; Article; (JOURNAL ARTICLE)
LANGUAGE: English
FILE SEGMENT: Priority Journals; Cancer Journals
ENTRY MONTH: 199110

AB The construction, synthesis and secretion of a genetically engineered **antibody**-cytokine **fusion** molecule is described. To target **tumor necrosis factor** (**TNF**) to tumor cells, recombinant **antibody** techniques were used to produce a Fab-like **antibody-TNF** conjugate. At the gene level, the heavy chain gene of an antitransferrin receptor **antibody** was **linked** to a synthetic **TNF** gene encoding human **TNF**. Transfection of the heavy chain-**TNF** gene into a myeloma derived cell line which was producing the light chain of the same **antibody**, allowed the isolation of a cell line secreting a **fusion** protein of the expected molecular weight and composition. The culture supernatant of the cell line contained **TNF** cytotoxic activity towards murine L929 cells and human MCF-7 cells. Cytotoxicity towards the human cancer cells was inhibited by an excess of the original antitransferrin receptor **antibody**, indicating that the **antibody-TNF** molecules are targeted to the transferrin receptor rich tumor cells. Since the **antibody** genes used are chimeric (i.e. composed of mouse variable and human constant regions) and since DNA encoding human **TNF** was used, the hybrid protein is an example of a humanized immunotoxin-like molecule. These results illustrate the possibilities of **antibody** engineering technology to create and produce improved agents for cancer therapy. Furthermore, they demonstrate for the first time the ability of myeloma cells to secrete an **antibody**-cytokine chimeric molecule.

MOLECULAR AND CELLULAR BIOLOGY, Mar. 1989, p. 1165–1172
0270-7306/89/031165-08$02.00/0

Vol. 9, No. 3

p185^{HER2} Monoclonal Antibody Has Antiproliferative Effects In Vitro and Sensitizes Human Breast Tumor Cells to Tumor Necrosis Factor

ROBERT M. HUDZIAK,[1] GAIL D. LEWIS,[2] MARCY WINGET,[3] BRIAN M. FENDLY,[3] H. MICHAEL SHEPARD,[2] AND AXEL ULLRICH[1]†*

Departments of Developmental Biology,[1] Pharmacological Sciences,[2] and Medicinal and Analytical Chemistry,[3] Genentech, Inc., 460 Point San Bruno Boulevard, South San Francisco, California 94080

Received 3 October 1988/Accepted 8 December 1988

The *HER2/c-erbB-2* gene encodes the epidermal growth factor receptorlike human homolog of the rat *neu* oncogene. Amplification of this gene in primary breast carcinomas has been shown to correlate with poor clinical prognosis for certain cancer patients. We show here that a monoclonal antibody directed against the extracellular domain of p185^{HER2} specifically inhibits the growth of breast tumor-derived cell lines overexpressing the *HER2/c-erbB-2* gene product and prevents *HER2/c-erbB-2*-transformed NIH 3T3 cells from forming colonies in soft agar. Furthermore, resistance to the cytotoxic effect of tumor necrosis factor alpha, which has been shown to be a consequence of *HER2/c-erbB-2* overexpression, is significantly reduced in the presence of this antibody.

HER2/c-erbB-2, the human homolog of the rat proto-oncogene *neu* (4, 34), encodes a 1,255-amino-acid glycoprotein with extensive homology to the human epidermal growth factor (EGF) receptor (4, 21, 33, 34, 42). The *HER2/c-erbB-2* gene product, p185^{HER2}, has all of the structural features and many of the functional properties of subclass I growth factor receptors (reviewed in references 43 and 44), including cell surface location and an intrinsic tyrosine kinase activity. However, the ligand for this putative growth factor receptor has not yet been identified.

Amplification of the *HER2/c-erbB-2* gene has been found in human salivary gland and gastric tumor-derived cell lines (13, 34), as well as in mammary gland carcinomas (21, 22, 40, 42). Slamon et al. (35) surveyed 189 primary breast adenocarcinomas and determined that the *HER2/c-erbB-2* gene was amplified in about 30% of the cases. Most importantly, *HER2/c-erbB-2* amplification was correlated with a negative prognosis and high probability of relapse. Similar although less frequent amplification of the *HER2/c-erbB-2* gene has been reported for gastric and colon adenocarcinomas (45, 46). Experiments with NIH 3T3 cells also suggest a direct role for the overexpressed, structurally unaltered *HER2/c-erbB-2* gene product p185^{HER2} in neoplastic transformation. High levels of *HER2/c-erbB-2* gene expression attained by coamplification of the introduced gene with dihydrofolate reductase by methotrexate selection (18) or by using a strong promoter (6) was shown to transform NIH 3T3 fibroblasts. Only cells with high levels of p185^{HER2} are transformed, i.e., have an altered morphology, are anchorage independent, and will form tumors in athymic mice.

Overexpression of p185^{HER2} may, furthermore, contribute to malignant tumor development by allowing tumor cells to evade one component of the antitumor defenses of the body, the activated macrophage (17). Macrophages play an important role in immune surveillance against neoplastic growth in vivo (1, 2, 38), and Urban et al. (39) have shown that tumor cells made resistant to macrophages display enhanced tumorigenicity. Tumor necrosis factor alpha (TNF-α) has been shown to play a role in activated macrophage-mediated tumor cell killing in vitro (3, 11, 23, 29, 39). NIH 3T3 cells transformed by a transfected and amplified *HER2/c-erbB-2* cDNA show increased resistance to the cytotoxic effects of activated macrophages or TNF-α in direct correlation with increased levels of p185^{HER2} expression. Furthermore, breast tumor cell lines with high levels of p185^{HER2} exhibit resistance to TNF-α. Resistance to host antitumor defenses could facilitate the escape of cells from a primary tumor to establish metastases at distant sites.

To further investigate the consequences of alteration in *HER2/c-erbB-2* gene expression in mammary gland neoplasia and to facilitate investigation of the normal biological role of the *HER2/c-erbB-2* gene product, we have prepared monoclonal antibodies against the extracellular domain of p185^{HER2}. One monoclonal antibody (4D5) was characterized in more detail and was shown to inhibit in vitro proliferation of human breast tumor cells overexpressing p185^{HER2} and, furthermore, to increase the sensitivity of these cells to the cytotoxic effects of TNF-α.

MATERIALS AND METHODS

Cells and cell culture. Human tumor cell lines were obtained from the American Type Culture Collection. The mouse fibroblast line NIH 3T3/HER2-3$_{400}$, expressing an amplified *HER2/c-erbB-2* cDNA under simian virus 40 early promoter control, and the vector-transfected control cell line NIH 3T3/CVN have been described previously (18).

Cells were cultured in a 1:1 mixture of Dulbecco modified Eagle medium and Ham nutrient mixture F-12 supplemented with 2 mM glutamine, 100 u of penicillin per ml, 100 μg of streptomycin per ml, and 10% serum. Human tumor cell lines were cultured with fetal bovine serum (GIBCO Laboratories, Grand Island, N.Y.); NIH 3T3 derivatives were cultured with calf serum (Hyclone Laboratories, Inc., Logan, Utah.).

Immunization. Female BALB/c mice were immunized with NIH 3T3/HER2-3$_{400}$ cells expressing high levels of

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$p185^{HER2}$. The cells were washed once with phosphate-buffered saline (PBS) and detached from the plate with PBS containing 25 mM EDTA. After low-speed centrifugation, the cells were suspended in cold PBS (2×10^7 cells per ml). Each mouse was injected intraperitoneally with 0.5 ml of this cell suspension on weeks 0, 2, 5, and 7.

On weeks 9 and 13, 100 μl of a Triton X-100 membrane preparation of $p185^{HER2}$, partially purified by wheat germ agglutinin chromatography (700 μg of protein per ml) (25), was administered intraperitoneally. Three days before fusion, 100 μl of the enriched $p185^{HER2}$ protein was administered intravenously.

Fusion and screening. Mice with high antibody titers as determined by immunoprecipitation of $p185^{HER2}$ were sacrificed, and their splenocytes were fused as described previously (26). Spleen cells were mixed at a 4:1 ratio with the fusion partner, mouse myeloma cell line X63-Ag8.653 (20), in the presence of 50% polyethylene glycol 4000. Fused cells were plated at a density of 2×10^5 cells per well in 96-well microdilution plates. The hypoxanthine-azaserine (12) selection for hybridomas was begun 24 h later. Beginning at day 10 postfusion, supernatants from hybridoma-containing wells were tested for the presence of antibodies specific for $p185^{HER2}$ by an enzyme-linked immunosorbent assay with the wheat germ agglutinin chromatography-purified $p185^{HER2}$ preparation (28). Enzyme-linked immunosorbent assay-positive supernatants were confirmed by immunoprecipitation and cloned twice by limiting dilution.

Large quantities of specific monoclonal antibodies were produced by preparation of ascites fluid; antibodies were then purified on protein A-Sepharose columns (Fermentech, Inc., Edinburgh, Scotland) and stored sterile in PBS at 4°C.

Immunoprecipitations and antibodies. Cells were harvested by trypsinization, counted in a Coulter counter (Coulter Electronics, Inc., Hialeah, Fla.), and plated 24 h before being harvested for analysis of $p185^{HER2}$ expression. Cells were lysed at 4°C with 0.8 ml of HNEG lysis buffer (18) per 100-mm plate. After 10 min, 1.6 ml of lysis dilution buffer (HNEG buffer with 1% bovine serum albumin and 0.1% Triton X-100) was added to each plate, and the extracts were clarified by centrifugation at 12,000 × *g* for 5 min.

Antibodies were added to the cell extracts and allowed to bind at 4°C for 2 to 4 h. Immune complexes were collected by adsorption to protein A-Sepharose beads for 20 min and washed three times with 1 ml of HNEG buffer–0.1% Triton X-100. Autophosphorylation reactions were carried out for 20 min at 4°C in 50 μl of HNEG wash buffer containing 5 mM $MnCl_2$ and 3 μCi of [γ-^{32}P]ATP (5,000 Ci/mmol, Amersham Corp., Arlington Heights, Ill.). The autophosphorylation reaction conditions have been described previously (18). Proteins were separated on sodium dodecyl sulfate (SDS)–7.5% polyacrylamide gels and analyzed by autoradiography.

The polyclonal antibody, G-H2CT17, recognizing the carboxy-terminal 17 amino acids of $p185^{HER2}$, has been described previously (18). The anti-EGF receptor monoclonal antibody 108 (16) was provided by Joseph Schlessinger, Rorer Biotechnology, Inc.

Fluorescence-activated cell sorting. SK-BR-3 human breast tumor cells overexpressing the *HER2/c-erbB-2* gene (17, 22) or A431 human squamous carcinoma cells overexpressing the EGF receptor gene (14) were grown in T175 flasks. They were detached from the flasks by treatment with 25 mM EDTA–0.15 M NaCl, collected by low-speed centrifugation, and suspended at 1×10^6 cells per ml in PBS–1% fetal bovine serum. One milliliter of each cell line was incubated with 10 μg of either anti-*HER2/c-erbB-2* monoclonal antibody (4D5) or a control antibody (40.1.H1) recognizing the hepatitis B surface antigen. The cells were washed twice and suspended on ice for 30 min in 1 ml of PBS–1% fetal bovine serum containing 10 μg of goat anti-mouse immunoglobulin G $F(ab')_2$ fragments conjugated with fluorescein isothiocyanate dye (Boehringer Mannheim Biochemicals, Indianapolis, Ind.). Unbound fluorescein dye was removed by two further washes. The cells were suspended at 2×10^6 per ml in PBS–1% fetal bovine serum and analyzed with an EPICS 753 (Coulter) fluorescence-activated cell sorter. Fluorescein was excited by 300 mW of 488-nm argon laser light, and the emitted light was collected with a 525-nm band-pass filter with a 10-nm band width.

Down-regulation assay. SK-BR-3 cells were plated at 1.5×10^5 cells per 35-mm culture dish in normal medium. After a 6-h period to allow attachment, the medium was replaced by 1.5 ml of methionine-free labeling medium containing 150 μCi of [^{35}S]methionine per ml and 2% dialyzed fetal bovine serum. The cells were metabolically labeled for 14 h and then chased with medium containing 2% dialyzed serum and unlabeled methionine. Either a control monoclonal antibody (40.1.H1) or anti-$p185^{HER2}$ (4D5) was added to a final concentration of 2.5 μg/ml. At 0, 5, and 11 h, extracts were prepared with 0.3 ml of lysis solution and 0.6 ml of dilution buffer. The $p185^{HER2}$ was immunoprecipitated with 2.5 μl of polyclonal antibody G-H2CT17. The washed immune complexes were dissolved in sample buffer, electrophoresed on a SDS-7.5% polyacrylamide gel, and analyzed by autoradiography. Each time point determination was performed in duplicate. Autoradiograph band intensities were quantitated by using a scanner (Ambis Systems).

Cell proliferation assays. The anti-$p185^{HER2}$ monoclonal antibodies were characterized by using the breast tumor cell line SK-BR-3. Cells were detached by using 0.25% (vol/vol) trypsin and suspended in complete medium at a density of 4

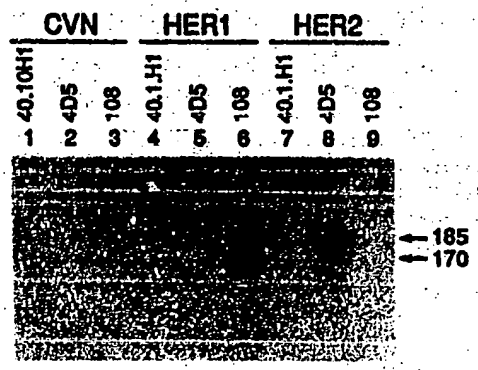

FIG. 1. Specificity of monoclonal antibody 4D5. Three cell lines, NIH 3T3/CVN, NIH 3T3/HER1-EGF receptor, and NIH 3T3/HER2-3_{400}, were plated out at 2.0×10^6 in 100-mm culture dishes. At 24 h, Triton X-100 lysates were prepared and divided into three portions. Either an irrelevant monoclonal antibody (6 μg of anti-hepatitis B virus surface antigen, 40.1.H1; lanes 1, 4, and 7), anti-$p185^{HER}$ monoclonal antibody 4D5 (6 μg; lanes 2, 5, and 8), or anti-EGF receptor monoclonal antibody 108 (6 μg; lanes 3, 6, and 9) was added and allowed to bind at 4°C for 4 h. The immune complexes were collected with 30 μl of protein A-Sepharose. Rabbit anti-mouse immunoglobulin (7 μg) was added to each 4D5 immunoprecipitation to improve the binding of this monoclonal antibody to the protein A-coated beads. Proteins were labeled by autophosphorylation and separated on an SDS–7.5% polyacrylamide gel. The gel was exposed to film at −70°C for 4 h with an intensifying screen. The arrows show the positions of proteins of M_r 185,000 and 170,000.

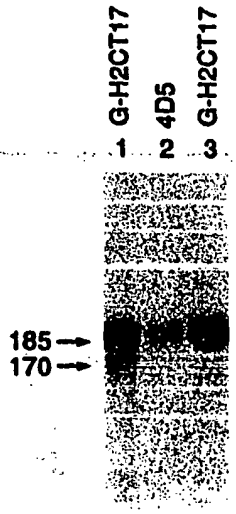

FIG. 2. Binding of monoclonal antibody 4D5 to unglycosylated receptor. NIH 3T3/HER2-3$_{400}$ cells were plated into two 100-mm plates at 2 × 10^6 cells per plate. After 14 h, the antibiotic tunicamycin was added to one plate at 3 μg/ml. After a further 5.5 h of incubation, Triton X-100 lysates were then prepared from each plate. Immunoprecipitations, the autophosphorylation reaction, and SDS-polyacrylamide gel electrophoresis were performed as described in the legend to Fig. 1. Lanes: 1, tunicamycin-treated cell lysate (one-third of a plate) immunoprecipitated with 2.5 μl of a polyclonal antibody directed against the C terminus of p185^{HER2}; 2, tunicamycin-treated cell lysate (one-third of a plate) immunoprecipitated with 6 μg of 4D5; 3, untreated control lysate (one-third of a plate) immunoprecipitated with the polyclonal antibody. The arrows show the locations of proteins of M_r 185,000 and 170,000.

× 10^5 cells per ml. Aliquots of 100 μl (4 × 10^4 cells) were plated into 96-well microdilution plates, the cells were allowed to adhere, and 100 μl of media alone or media containing monoclonal antibody (final concentration, 5 μg/ml) was then added. After 72 h, plates were washed twice with PBS (pH 7.5), stained with crystal violet (0.5% in methanol), and analyzed for relative cell proliferation as described previously (36).

For assays in which monoclonal antibodies were combined with recombinant human TNF-α (5.0 × 10^7 U/mg; Genentech, Inc.), cells were plated and allowed to adhere as described above. Following cell adherence, control medium alone or medium containing monoclonal antibodies was added to a final concentration of 5 μg/ml. Cultures were incubated for another 4 h, and then increasing concentrations of TNF-α were added to a final volume of 200 μl. Following 72 h of incubation, the relative cell number was determined by crystal violet staining. Some samples were analyzed by crystal violet staining following cell adherence for determination of the initial cell number.

RESULTS

Specificity of monoclonal antibody 4D5. Monoclonal antibodies directed against the extracellular domain of p185^{HER2} were prepared by immunizing mice with NIH 3T3 cells transfected with a *HER2/c-erbB-2* cDNA (HER2-3$_{400}$) (17, 18) and overexpressing the corresponding gene product, p185^{HER2}. One antibody exhibited several interesting biological properties and was chosen for further characterization. Antibody 4D5 specifically immunoprecipitated a single ^{32}P-labeled protein of M_r 185,000 from NIH 3T3 cells expressing p185^{HER2} (Fig. 1, lane 8). This antibody did not cross-react with the human EGF receptor (HER1; Fig. 1, lane 5), even when overexpressed in a mouse NIH 3T3 background (Fig. 1, lane 6). Furthermore, it did not immunoprecipitate any proteins from NIH 3T3 cells transfected with a control plasmid (pCVN) which expresses the neomycin resistance and dihydrofolate reductase genes only (Fig. 1, lane 2).

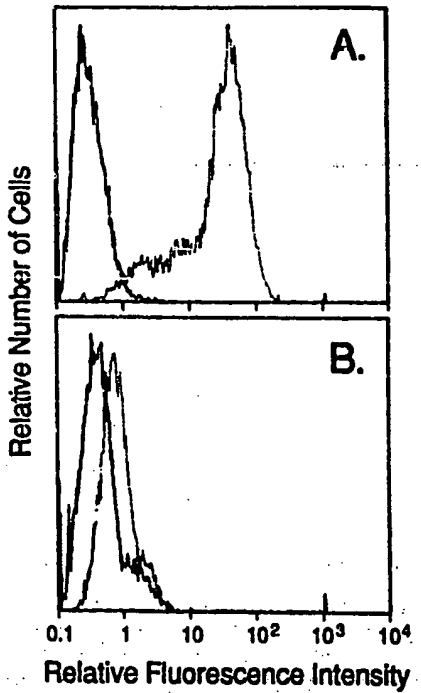

FIG. 3. Fluorescence-activated cell sorter histograms of human tumor cells binding anti-p185 monoclonal antibody 4D5. ——, Binding by the control antibody, 40.1.H1, directed against the hepatitis B surface antigen; ·····, binding by the anti-*HER2/c-erbB-2* antibody, 4D5. The antibodies were first allowed to react with the cell surface. After a wash step, bound antibody was labeled by addition of fluorescein-conjugated F(ab')$_2$ fragment of goat anti-mouse immunoglobulin G. (A) Binding of the antibodies to the human breast tumor line SK-BR-3, which contains an amplification of the *HER2/c-erbB-2* gene and expresses high levels of the *HER2/c-erbB-2* gene product p185^{HER2}. (B) Binding of the same antibodies to the human squamous epithelial cell line A431. This cell line expresses low levels of mRNA for *HER2/c-erbB-2* and high levels (2 × 10^6 receptors per cell) of the EGF receptor.

To determine the nature of the epitope recognized by 4D5, NIH 3T3/HER2-3$_{400}$ cells were treated with tunicamycin, which prevents addition of N-linked oligosaccharides to proteins (15, 41). Cells treated with this antibiotic for 5.5 h contained two proteins which were immunoprecipitated by a polyclonal antibody against the carboxy-terminal peptide of p185^{HER2} (Fig. 2, lane 1). The polypeptide of 170,000 M_r represents unglycosylated p185^{HER2}. The upper band of ca. 185,000 M_r comigrated with glycosylated p185^{HER2} from untreated cells (Fig. 2, lane 3). Monoclonal antibody 4D5 efficiently immunoprecipitated only the glycosylated form of p185^{HER2} (Fig. 2, lane 2). This experiment suggests either that the epitope recognized by 4D5 consists partly of carbohydrate, or, alternatively, that the antibody recognizes a conformation of the protein achieved only when it is glycosylated.

TABLE 1. Inhibition of SK-BR-3 proliferation by anti-p185^{HER2} monoclonal antibodies[a]

Monoclonal antibody	Relative cell proliferation[b]
7C2	79.3 ± 2.2
2C4	79.5 ± 4.4
7D3	83.8 ± 5.9
4D5	44.2 ± 4.4
3E8	66.2 ± 2.4
6E9	98.9 ± 3.6
7F3	62.1 ± 1.4
3H4	66.5 ± 3.9
2H11	92.9 ± 4.8
40.1.H1	105.8 ± 3.8
4F4	94.7 ± 2.8

[a] SK-BR-3 breast tumor cells were plated as described in Materials and Methods. Following adherence, medium containing 5 μg of either anti-p185^{HER2} or control monoclonal antibodies (40.1.H1 and 4F4) per ml were added.

[b] Relative cell proliferation was determined by crystal violet staining of the monolayers after 72 h. Values are expressed as a percentage of results with untreated control cultures (100%).

The binding of monoclonal antibody 4D5 to human tumor cell lines was investigated by fluorescence-activated cell sorting (Fig. 3). This antibody was bound to the surface of cells expressing p185^{HER2}. Figure 3A shows the 160-fold increase in cellular fluorescence observed when 4D5 was added to SK-BR-3 breast adenocarcinoma cells relative to a control monoclonal antibody. This cell line contains an amplified *HER2/c-erbB-2* gene and expresses high levels of p185^{HER2} (17, 22). In contrast, the squamous carcinoma cell line A431, which expresses about 2×10^6 EGF receptors per cell (14) but only low levels of p185^{HER2} (4), exhibited only a twofold increase in fluorescence with 4D5 (Fig. 3B) when compared with a control monoclonal antibody.

The binding of 4D5 correlated with the levels of p185^{HER2} expressed by these two cell lines. SK-BR-3 cells, expressing high levels of p185^{HER2}, showed an 80-fold increase in relative fluorescence intensity compared with A431 cells. This experiment demonstrates that 4D5 specifically recognizes the extracellular domain of p185^{HER2}.

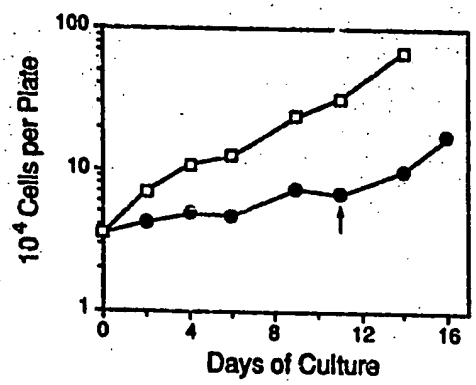

FIG. 4. Growth curve of SK-BR-3 cells treated with anti-*HER2/c-erbB-2* monoclonal antibody 4D5. Cells were plated into 35-mm culture dishes at 20,000 cells per plate in medium containing 2.5 μg of either control antibody (40.1.H1, anti-hepatitis B surface antigen) (□) or anti-p185^{HER2} antibody 4D5 (●) per ml. On the indicated days, cells were trypsinized and counted in a Coulter counter. The determination for each time point and each antibody was done in duplicate, and the counts were averaged. The arrow indicates the day the cells were refed with medium without antibodies.

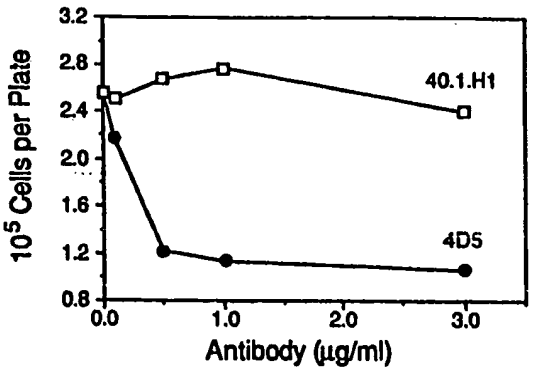

FIG. 5. Growth of SK-BR-3 cells in different concentrations of monoclonal antibody 4D5. The human breast tumor line SK-BR-3 was plated into 35-mm culture dishes at 20,000 cells per dish. Either 0.1, 0.5, 1.0, or 3.0 μg of a control monoclonal antibody (40.1.H1, anti-hepatitis B surface antigen) or monoclonal 4D5 antibody per ml was added at the time of plating. After 8 days of growth, the plates were trypsinized and the cells were counted in a Coulter counter. Each concentration of antibody was plated and counted in duplicate, and the cell numbers were averaged.

Effects on cell proliferation. We used the human mammary gland adenocarcinoma cell line, SK-BR-3, to determine whether monoclonal antibodies directed against the extracellular domain of p185^{HER2} had any effect on the proliferation of cell lines overexpressing this receptorlike protein. SK-BR-3 cells were coincubated with several *HER2/c-erbB-2*-specific monoclonal antibodies or with either of two different control monoclonal antibodies (40.1.H1, directed against the hepatitis B surface antigen; 4F4, directed against recombinant human gamma interferon). Most anti-*HER2/c-erbB-2* monoclonal antibodies which recognize the extracellular domain inhibited the growth of SK-BR-3 cells (Table

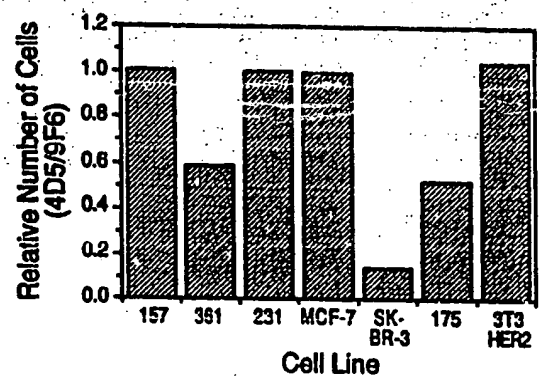

FIG. 6. Screening of breast tumor cell lines for growth inhibition by monoclonal antibody 4D5. Each cell line was plated in 35-mm culture dishes at 20,000 cells per dish. Either a control monoclonal antibody (9F6, anti-human immunodeficiency virus gp120) or the anti-p185^{HER2} monoclonal antibody 4D5 was added on day 0 to 2.5 μg/ml. Because the different cell lines grow at different rates, the cell lines NIH 3T3/HER2-3$_{400}$ and SK-BR-3 were counted after 6 days, cell lines MDA-MB-157, MDA-MB-231, and MCF-7 were counted after 9 days, and cell lines MDA-MB-175VII and MDA-MB-361 were counted after 14 days. The difference in growth between cells treated with 4D5 and 40.1.H1 is expressed as the ratio of cell numbers with 4D5 versus a control monoclonal antibody, 9F6. Each cell line was assayed in duplicate for each antibody, and the counts were averaged.

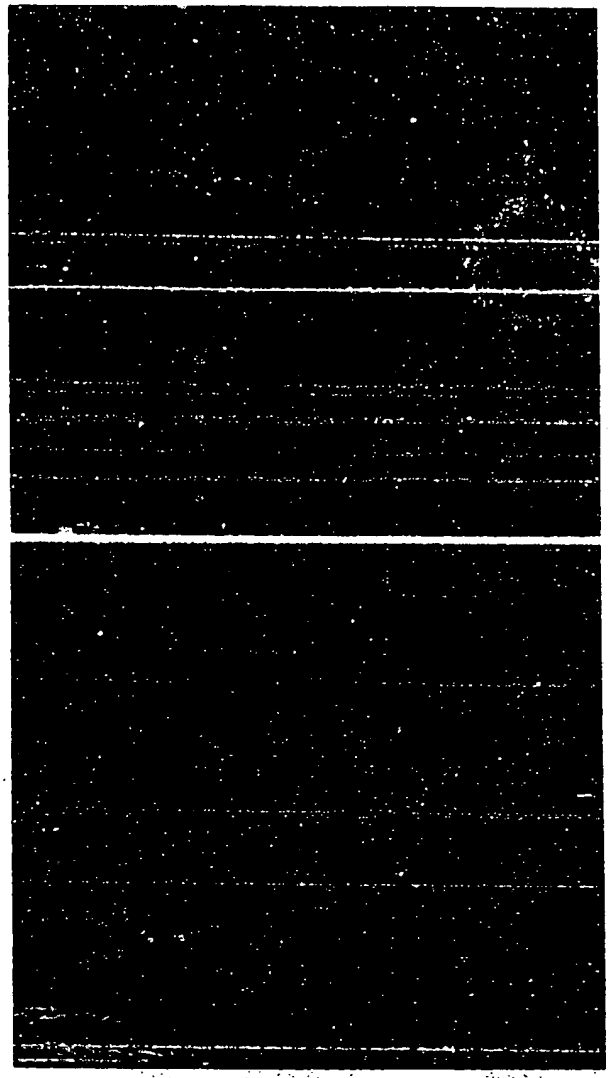

FIG. 7. Inhibition of anchorage-independent growth of NIH 3T3/HER2-3_{400} cells by 4D5. Cells (20,000 per 60-mm plate) were plated in 0.2% soft agar over a 0.4% agar base. After 3 weeks, the plates were photographed at ×100 magnification by using a Nikon microscope with phase-contrast optics. (a) HER2-3_{400} cells plated in agar containing 200 ng of a control antibody (TF-C8) per ml. (b) The same cells plated in agar containing 200 ng of 4D5 per ml.

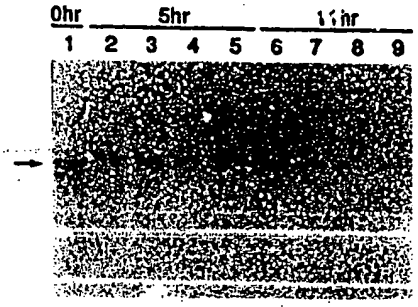

FIG. 8. Effect of antibody binding on p185HER2 turnover. SK-BR-3 cells were labeled for 14 h with [^{35}S]methionine. The label was then chased with cold methionine and either an irrelevant monoclonal antibody (40.1.H1, anti-hepatitis B surface antigen) or 4D5 was added to 2.5 μg/ml. The cells on the plates were lysed at 0, 5, and 11 h, and ^{35}S-labeled p185HER2 was quantitated by immunoprecipitation with the C-terminal specific polyclonal antibody. The 5- and 11-h time point determinations were performed in duplicate for each of the two antibodies. Proteins were separated by SDS-polyacrylamide gel electrophoresis. The fluor-treated gel was exposed to film for 4 h at room temperature. The arrow indicates the position of a protein of M_r 185,000. Band intensities were quantitated by using an Ambis Systems scanner. Lanes: 1, 0 h; lanes 2 and 3, 40.1.H1 (5 h); lanes 4 and 5, 4D5 (5 h); lanes 6 and 7, 40.1.H1 (11 h); lanes 8 and 9, 4D5 (11 h).

1). Maximum inhibition was obtained with monoclonal antibody 4D5, which inhibited cellular proliferation by 56%. The control antibodies had no significant effect on cell growth.

Figure 4 compares the growth of SK-BR-3 cells in the presence of either a control antibody, 40.1.H1, or the anti-p185HER2 antibody. Proliferation of the cells was inhibited when antibody 4D5 was present. The generation time increased from 3.2 to 12.2 days. To determine whether 4D5 treatment was cytostatic or cytotoxic, antibody was removed by medium change 11 days after treatment. The cells resumed growth at a nearly normal rate, suggesting that the antibody affected cell growth rather than cell viability. The dose-response curve (Fig. 5) showed that a concentration of 200 ng/ml inhibited growth by 50%, whereas maximum effects were achieved by using a concentration of between 0.5 and 1 μg/ml.

The effect of 4D5 on the proliferation of six additional breast tumor cell lines, as well as mouse NIH 3T3 fibroblasts transformed by p185HER2 overexpression (NIH 3T3/HER2-3_{400}), was tested in monolayer growth assays. Cells were plated at low density in medium containing 2.5 μg of either a control antibody or 4D5 per ml. When the cultures approached confluency, cells were removed with trypsin and counted. 4D5 did not have any significant effect on the growth of the MCF-7, MDA-MB-157, MDA-MB-231, or NIH 3T3/HER2-3_{400} cell lines (Fig. 6). It did, however, significantly affect the growth of the cell lines MDA-MB-361 (58% of control) and MDA-MB-175-VII (52% of control), which express high levels of p185HER2 (17).

Interestingly, monoclonal antibody 4D5 had no effect on the monolayer growth of the NIH 3T3/HER2-3_{400} cell line. However, it completely prevented colony formation by these cells in soft agar (Fig. 7), a property which had been induced by *HER2/c-erbB-2* amplification (18). In the presence of 200 ng of a control monoclonal antibody (antitissue factor, TC-C8) per ml, 116 (average of two plates) soft-agar colonies were counted, while the same cells plated simultaneously into soft agar containing 200 ng of 4D5 per ml did not yield any colonies.

Monoclonal antibody 4D5 down-regulates p185HER2. To determine whether the antiproliferative effect of 4D5 was due to enhanced degradation of p185HER2, we measured its rate of turnover in the presence or absence of antibody. p185HER2 was metabolically labeled by culturing SK-BR-3 cells for 14 h in the presence of [^{35}S]methionine. Cells were then chased for various times, and either a control antibody or 4D5 was added at the beginning of the chase period. At 0, 5, and 11 h, cells were lysed and p185HER2 levels were assayed by immunoprecipitation and SDS-polyacrylamide gel electrophoresis. p185HER2 is degraded more rapidly after exposure of SK-BR-3 cells to 4D5 (Fig. 8). Densitometric evaluation of the data showed that the p185HER2 half-life of

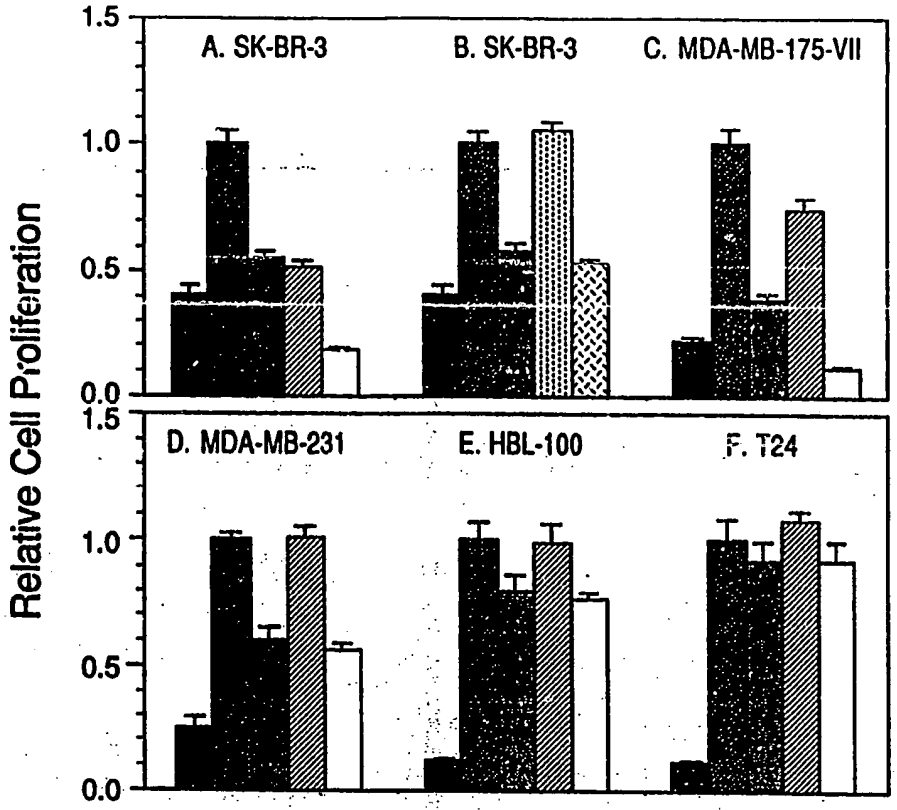

FIG. 9. Monoclonal antibody 4D5 sensitizes breast tumor cells to the cytotoxic effects of TNF-α. Cells were plated in 96-well microdilution plates (4 × 10^4 cells per well for SK-BR-3, MDA-MB-175-VII, and MDA-MB-231; 10^4 cells per well for HBL-100 and T24) and allowed to adhere for 2 h. Anti-*HER2*/c-*erbB*-2 monoclonal antibody 4D5 (5 μg/ml) or anti-hepatitis B surface antigen monoclonal antibody 40.1.H1 (5 μg/ml) was then added for a 4-h incubation prior to the addition of TNF-α to a final concentration of 10^4 units/ml. After 72 h, the monolayers were washed twice with PBS and stained with crystal violet dye for determination of relative cell proliferation. In addition, some cell monolayers were stained with crystal violet following adherence in order to determine the initial cell density for comparison with cell densities measured after 72 h. The symbols denote initial cell density (■), untreated (control) cells (■), cells treated with TNF-α (■), 4D5 (▨), TNF-α plus 4D5 (□), 40.1.H1 (▨); or TNF-α plus 40.1.HI (▨).

7 h decreased to 5 h in the presence of antibody (data not shown).

Monoclonal antibody 4D5 enhances TNF-α cytotoxicity. The addition of certain growth factors to tumor cells has been shown to increase their resistance to the cytotoxic effects of TNF-α (37). A prediction based on these findings would be that expression of oncogenes that mimic or replace growth factor receptor function may also increase the resistance of cells to this cytokine. Recently, it was shown that overexpression of the putative growth factor receptor $p185^{HER2}$ in NIH 3T3 cells caused an increase in the resistance of these cells to TNF-α (17). Furthermore, breast tumor cell lines with high levels of $p185^{HER2}$ also exhibited TNF-α resistance.

To further investigate the mechanism by which the 4D5 antibody inhibited cell growth, we investigated the response of three breast tumor cell lines to TNF-α in the presence or absence of this antibody. If the anti-$p185^{HER2}$ monoclonal antibody 4D5 inhibited proliferation of breast tumor cells by interfering with the signalling functions of $p185^{HER2}$, addition of this antibody would be expected to enhance the sensitivity of tumor cells to TNF-α. Both SK-BR-3 (Fig. 9A) and MDA-MB-175-VII (Fig. 9C) were growth inhibited by both the monoclonal antibody 4D5 (5 μg/ml; 50% and 25% inhibition, respectively) and high concentrations of TNF-α (1 × 10^4 units/ml; 50% and 60% inhibition, respectively). However, the combination of TNF-α and monoclonal antibody 4D5 reduced the SK-BR-3 and MDA-MB-175-VII tumor cell number to a level below that initially plated, indicating the induction of a cytotoxic response. In a separate experiment, SK-BR-3 cell viability was determined directly by using trypan blue dye exclusion, yielding identical results to those described above that were obtained by using crystal violet staining (data not shown). A control monoclonal antibody, 40.1.H1, did not inhibit SK-BR-3 breast tumor cell proliferation, nor did it induce an enhanced sensitivity of this cell line to the cytotoxic effects of TNF-α (Fig. 9B). In addition, the growth of the breast tumor cell line MDA-MB-231, which does not express detectable levels of $p185^{HER2}$ (17), was unaffected by monoclonal antibody 4D5, and the growth inhibition seen with the combination of 4D5 and TNF-α was similar to that observed with TNF-α alone (Fig. 9D). Furthermore, neither HBL-100 (30), a nontransformed but immortalized human breast epithelial cell line (Fig. 9E), nor T24 (27), a human bladder carcinoma cell line (Fig. 9F), expressed high levels of $p185^{HER2}$ (data not shown), and neither demonstrated growth inhibition by 4D5 or an enhanced growth-inhibitory or cytotoxic response to the combination of TNF-α and monoclonal antibody 4D5. These results demonstrate that only tumor cells which

overexpress p185^{HER2} will become sensitized to the cytotoxic effects of TNF-α by antibody 4D5.

DISCUSSION

We have prepared monoclonal antibodies against the extracellular domain of the *HER2*/c-*erbB*-2 gene product, p185^{HER2}, and have found that one of these, 4D5, strongly inhibits the growth of several breast tumor cell lines and furthermore sensitizes p185^{HER2}-overexpressing breast carcinoma cell lines SK-BR-3 and MDA-MB-175-VII to the cytotoxic effects of TNF-α. Monoclonal antibody 4D5 is specific for p185^{HER2} and shows no cross-reactivity with the closely related human EGF receptor expressed in mouse fibroblasts. Of six mammary carcinoma cell lines tested, only the three lines which express high levels of p185^{HER2} (SK-BR3, MBA-MB-175, and MDA-MD-361 [17]) were growth inhibited, and 4D5 did not inhibit the proliferation of a nontransformed human breast epithelial cell line, HBL-100, or the bladder carcinoma cell line T24.

In the presence of the antibody, the inhibition of SK-BR-3 cell growth was nearly complete, but the effect was cytostatic rather than cytotoxic. This property of 4D5 is similar to that described for a subset of monoclonal antibodies to the EGF receptor (19, 31, 32) which inhibit the growth of A431 cells, a human squamous epithelial carcinoma line expressing high levels of the EGF receptor. In this case, these inhibitory antibodies compete with radiolabeled EGF for binding to the receptor, and antibodies which do not block EGF binding have no effect on A431 cell growth. It has been suggested (J. Mendelsohn and H. Masui, Clin. Res. 35:600A, 1987) that these antibodies inhibit cell growth by interfering with an autocrine system involving the EGF receptor and an essential growth factor, transforming growth factor alpha, that is produced by the cells (5). It is therefore intriguing to speculate that antibody 4D5 analogously interferes with ligand binding to the *HER2*/c-*erbB*-2 gene product. Since an appropriate ligand for the putative *HER2*/c-*erbB*-2 receptor has not yet been identified, this possibility cannot yet be tested directly.

The 4D5 antibody had no effect on the growth of NIH 3T3 cells transformed by *HER2*/c-*erbB*-2 overexpression. However, it reversed one property conferred on these cells by amplification of the *HER2*/c-*erbB*-2 cDNA: the formation of colonies in soft agar was prevented by 200 ng of 4D5 antibody per ml. This result is similar to those obtained by Drebin et al. (8) with a monoclonal antibody to the rat *neu* oncogene-encoded p185^{neu}. They also observed that an anti-p185^{neu} monoclonal antibody inhibited colony growth in soft agar and tumor formation by *neu*-transformed NIH 3T3 cells in athymic mice (7–10). This effect was attributed to a lowering p185^{neu} levels by an increase in receptor turnover triggered by antibody binding. The apparent discrepancy between 4D5 effects on proliferation of breast tumor cells versus transfected mouse fibroblast cells is most probably a reflection of the fact that SK-BR-3 cells are authentic cancer cells, in contrast to the NIH 3T3 model system. Whereas SK-BR-3 cells may have evolved to be dependent on *HER2*/c-*erbB*-2-mediated signals for both growth and transformation characteristics, NIH 3T3 cells have acquired a transformed phenotype only as a result of *HER2*/c-*erbB*-2 overexpression, but may proliferate normally in response to other serum growth factors, even in the presence of blocking anti-p185^{HER2} antibody.

Previous work has shown that high-level expression of p185^{HER2} will transform NIH 3T3 cells and has suggested a casual role for amplification of the *HER2*/c-*erbB*-2 gene in mammary gland neoplasia. We have shown here that *HER2*/c-*erbB*-2 gene overexpression in NIH 3T3 cells is associated with increased resistance to the monokine TNF-α and that breast tumor cell lines which overexpress p185^{HER2} are resistant to the cytotoxic effects of TNF-α. The mechanism by which 4D5 inhibits breast tumor cell proliferation and reverses phenotypes associated with high levels of p185^{HER2} expression, such as resistance to TNF-α, is not clear. However, these results suggest that in addition to its ability to transform cells by virtue of overexpression (6, 18), *HER2*/c-*erbB*-2 could play a role in tumor progression by allowing tumor cells overexpressing p185^{HER2} to evade one component of the antitumor immunosurveillance of the host, the activated macrophage (17). These properties of the *HER2*/c-*erbB*-2 gene product may in part explain the aggressive, single-step induction of mammary adenocarcinoma in transgenic mice bearing the *neu* oncogene (24), which encodes the mutated rat homolog of p185^{HER2}.

The experiments presented here demonstrate that a monoclonal antibody which recognizes the extracellular domain of p185^{HER2} inhibits the proliferation of breast tumor cells which overexpress this receptorlike protein. Moreover, treatment with this antibody also sensitizes these tumor cells to the cytotoxic effects of TNF-α. Monoclonal antibodies specific for p185^{HER2} may therefore be useful therapeutic agents for the treatment of human neoplasias, including certain mammary carcinomas, which are characterized by the overexpressing of p185^{HER2}

ACKNOWLEDGMENTS

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[54] **RECOMBINANT ANTIBODIES SPECIFIC FOR A GROWTH FACTOR RECEPTOR**

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[58] Field of Search 435/69.7, 252.3; 425/320.1; 530/350, 387.3; 536/23.4

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[57] **ABSTRACT**

The invention concerns recombinant antibodies directed to the extracellular domain of the human growth factor receptor c-erbB-2 comprising a light chain variable domain and a heavy chain variable domain of a monoclonal antibody, monoclonal antibodies directed to c-erbB-2 themselves, a method of manufacturing those recombinant and monoclonal antibodies, hybridoma cells secreting those monoclonal antibodies, a method of manufacturing those hybridoma cells, DNAs encoding the heavy and light chain variable domains and the recombinant antibody, a method of manufacturing that DNA, hybrid vectors suitable for the expression of that DNA, host cells transformed with that DNA, and processes of using those recombinant and monoclonal antibodies in the diagnosis and treatment of tumors.

19 Claims, No Drawings

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RECOMBINANT ANTIBODIES SPECIFIC FOR A GROWTH FACTOR RECEPTOR

This is a continuation of Ser. No. 07/828,832, filed Jan. 31, 1992, abandoned, which is a continuation-in-part of Ser. No. 07/731,190, filed Jul. 15, 1991, now abandoned.

BACKGROUND OF THE INVENTION

Growth factors and their receptors are involved in the regulation of cell proliferation, and they also seem to play a role in tumor growth. The c-erbB-2 growth factor receptor protein, a protein of the membrane receptor protein tyrosine kinase family (A. Ullrich & J. Schlessinger, Cell 61: 203–212, 1990), is found in human breast tumors and human ovarian carcinomas. Amplification of the c-erbB-2 gene and over-expression of the protein appears to correlate with poor prognosis for tumor patients. Thus the c-erbB-2 protein has potential, both as a diagnostic marker and as a target for cancer therapy. Sequence analysis reveals that c-erbB-2, also called HER2, a glycoprotein of 185 kilo-Dalton (gp185), is identical or closely related to the human analog of the neu oncogene (A. L. Schechter et al., Science 229: 976–978, 1985) and shows considerable sequence homology to the human epidermal growth factor (EGF) receptor.

Of particular interest in tumor diagnosis and therapy are antibodies directed to tumor markers. Polyclonal antibodies may be obtained from the serum of mammals immunized with the antigen, i.e. the tumor marker. The development of hybridoma technology made it possible to generate continuous cell lines, in particular murine hybridomas, producing monoclonal antibodies of the desired specificity. Murine monoclonal antibodies directed to c-erbB-2 are known and are described, for example, by S. J. McKenzie at al., Oncogene 4: 543–548, 1989; R. M. Hudziak et al., Molecular and Cellular Biology 9: 1165–1172, 1989; International Patent Application WO 89/06692 (Genentech); and Japanese Patent Application Kokai 02-150 293 (Ajinomoto KK).

A major limitation in the use of murine-derived monoclonal antibodies as in vivo diagnostic and therapeutic agents is their immunogenicity as foreign proteins, their rather long persistence in the circulation, and the formation of damaging immune complexes. On the other hand, the treatment with human monoclonal antibodies is also limited since human hybridoma cell lines are hard to prepare, generally unstable, and do not produce monoclonal antibodies of appropriate specificity in sufficient quantities and at reasonable costs. In principle, the in vitro use of murine monoclonal antibodies is without limitation. However, production costs of monoclonal antibodies and, depending on the type of immunoassay used, the need for attaching a detectable marker to the antibody make it desirable to find more economic alternatives to regular murine monoclonal antibodies.

A promising alternative is the modification of immunoglobulin genes in order to tailor antibodies for particular diagnostic and therapeutic tasks. Due to the fact that the variable region and each of the constant region domains of immunoglobulin molecules are encoded in separate exons with their own splice sites, recombinant DNA techniques can be used to isolate different parts of cloned immunoglobulin genes and ligate them to parts of other immunoglobulins or to effector molecules. The reconstructed genes are expressed by appropriate transformed continuous cell lines. Murine antibodies can, for example, be converted into "humanized" antibodies by exchanging murine constant

domain exons for human immunoglobulin constant domain exons, thus generating chimeric antibodies with murine antibody-combining sites and human constant domains. The chimeric antibodies retain the antigen specificity determined by the murine variable domains, but also exhibit human effector functions (such as complement binding, stimulation of phagocytosis, triggering of granule release by mast cells) determined by the carboxy-terminal constant domain segments of the heavy chain polypeptides. An even more sophisticated technique in tailoring antibodies described in European Patent Application 0 239 400 exchanges also other fairly conserved domains, the so-called framework regions (FRs), within the murine variable domains for corresponding framework regions from human antibodies or for other human protein sequences. Such an antibody should be even less immunogenic in man since the only parts derived from a murine antibody are those hypervariable regions which define a particular specificity for an antigen, the so-called complementarity determining regions (CDRs).

Furthermore, fusion proteins different from immunoglobulins may be formed, e.g. single-chain antibodies, which retain the specificity and binding properties of the starting murine monoclonal antibody, but have otherwise novel properties derived from the non-immunoglobulin part of the fusion protein. The smallest domain of a monoclonal antibody which can bind to the antigen is the so-called Fv fragment which consists of the variable domains of the heavy and light chains. Fv fragments are difficult to prepare by proteolytic techniques since the corresponding variable domains tend to dissociate upon dilution. Fv molecules constructed by joining the variable domains of the heavy and light chains via a short peptide linker, also called single-chain antigen binding proteins, bind to an antigen with similar characteristics as the original monoclonal antibody (R. E. Bird et al., Science 242: 423–426, 1988; J. S. Huston et al., Proc. Natl. Acad. Sci. USA 85: 5879–5883, 1988; and International Patent Application WO 89/09825 (Celltech)). Fv encoding genes can, in principle, be linked to genes encoding effector molecules by recombinant gene technology. It is known, for example, that Fv encoding gene sequences can be linked to a gene encoding a portion of the Pseudomonas exotoxin A gene (V. K. Chaudhary et al., Nature 339: 394–397, 1989; and International Patent Application WO 89/11533 (I. Pastan et al.)).

OBJECT OF THE INVENTION

It is an object of this invention to provide recombinant antibodies directed to the extracellular domain of the human growth factor receptor c-erbB-2 comprising a light chain variable domain and a heavy chain variable domain of a monoclonal antibody, monoclonal antibodies directed to c-erbB-2 themselves, a method of manufacture of said recombinant antibodies and said monoclonal antibodies, hybridoma cells secreting said monoclonal antibodies, a method of manufacture of said hybridoma cells, DNA coding for the heavy chain variable domain, for the light chain variable domain and for the recombinant antibody, a method of manufacture of said DNA, hybrid vectors suitable for expression of said DNA, host cells transformed with said DNA, and the use of said recombinant antibodies and said monoclonal antibodies in the diagnosis and treatment of tumors.

DETAILED DESCRIPTION OF THE INVENTION

The invention concerns a recombinant antibody directed to the extracellular domain of the growth factor receptor

c-erbB-2, a human glycoprotein of 185 kilo-Dalton (gp185), comprising a heavy chain variable domain and a light chain variable domain of a monoclonal antibody.

Such a recombinant antibody may be a chimeric antibody consisting, for example, of a mouse heavy chain variable domain with the specificity for c-erbB-2 and a human heavy chain constant domain α, γ, δ, ε or μ, preferably γ, such as γ1 or γ4, and of a mouse light chain variable domain with the specificity for c-erbB-2 and a human light chain constant domain κ or λ, preferably κ, all assembled to give a functional antibody.

The preferred recombinant antibody of the invention is a single-chain antibody wherein the heavy chain variable domain and the light chain variable domain are linked by way of a spacer group, preferably a peptide. Most preferred is a single-chain antibody wherein the heavy chain variable domain is located at the N-terminus of the recombinant antibody. The single-chain recombinant antibody may further comprise an effector molecule and/or signal sequences facilitating the processing of the antibody by the host cell in which it is prepared. Effector molecules considered are those useful for diagnostic or therapeutic purposes, for example enzymes causing a detectable reaction, e.g. phosphatase, such as alkaline phosphatase from *E. coli* or mamalian alkaline phosphatase, e.g. bovine alkaline phosphatase, horseradish peroxidase, β-D-galactosidase, glucose oxidase, glucoamylase, carbonic anhydrase, acetylcholinesterase, lysozyme, malate dehydrogenase or glucose-6-phosphate, a peptide having particular binding properties, e.g. streptavidin from *Streptomyces avidinii* strongly binding to biotin, or enzymes, toxins or other drugs attacking the cells to which the antibody is bound, e.g. a protease, a cytolysin or an exotoxin, for example ricin A, diphtheria toxin A, or Pseudomonas exotoxin. In the following a single-chain recombinant antibody further comprising an effector molecule is referred to as fusion protein or intended to be within the meaning of the terms "single chain (recombinant) antibody" or "recombinant antibody", if appropriate.

The term effector molecule also includes biologically active variants of the above-mentioned proteins, e.g. variants produced from a DNA which has been subjected to in vitro mutagenesis, with the provision that the protein encoded by said DNA retains the biological activity of the native protein. Such modifications may consist in an addition, exchange or deletion of amino acids, the latter resulting in shortened variants. For example, an enzyme, such as phosphatase, may be prepared from a DNA which has been modified to facilitate the cloning of the encoding gene, or an exotoxin, such as Pseudomonas exotoxin, may be prepared from a DNA which has been mutated to delete the cell binding domain.

The recombinant antibodies of the invention are tested for their specificity to the extracellular domain of c-erbB-2, for example by immunofluorescent staining of cells expressing high levels of c-erbB-2, by immunoblotting either directly or by way of immunoprecipitation and protein blotting of the immunocomplexes, or by another immunoassay such as a binding, crossinhibition or competition radio- or enzyme immunoassay.

The variable domain of an antibody heavy or light chain consists of so-called framework regions (FRs), which are fairly conserved in antibodies with different specificities, and of hypervariable regions also called complementarity determining regions (CDRs), which are typical for a particular specificity.

Preferred recombinant antibodies of the invention are those wherein the heavy chain variable domain comprises a polypeptide of the formula

$$FR_1\text{-}CDR_{1H}\text{-}FR_2\text{-}CDR_{2H}\text{-}FR_3\text{-}CDR_{3H}\text{-}FR_4 \quad (I)$$

wherein FR_1 is a polypeptide residue comprising at least 25–29, preferably 25–33 naturally occurring amino acids, FR_2 is a polypeptide residue comprising 12–16 naturally occurring amino acids, FR_3 is a polypeptide residue comprising 30–34 naturally occurring amino acids, FR_4 is a polypeptide residue comprising at least 6–10, preferably 6–13 naturally occurring amino acids, CDR_{1H} is a polypeptide residue of the amino acid sequence 32 to 36 of SEQ ID NO:4 and 5, CDR_{2H} is a polypeptide residue of the amino acid sequence 51 to 67 of SEQ ID NO:4 and 5, and CDR_{3H} is a polypeptide residue of the amino acid sequence 100 to 109 of SEQ ID NO:4 and 5, or, CDR_{1H} is a polypeptide residue of the amino acid sequence 32 to 36 of SEQ ID NO:10 and 11, CDR_{2H} is a polypeptide residue of the amino acid sequence 51 to 67 of SEQ ID NO:10 and 11, and CDR_{3H} is a polypeptide residue of the amino acid sequence 100 to 110 of SEQ ID NO:10 and 11, and wherein the amino acid Cys may be in the oxidized state forming S—S-bridges. These particular complementarity determining regions are Asn-Tyr-Gly-Met-Asn (CDR_{1H}), Trp-Ile-Asn-Thr-Ser-Thr-Gly-Glu-Ser-Thr-Phe-Ala-Asp-Asp-Phe-Lys-Gly (CDR_{2H}), and Trp-Glu-Val-Tyr-His-Gly-Tyr-Val-Pro-Tyr (CDR_{3H}) according to SEQ. ID NO:4 and 5, or Ser-Tyr-Trp-Met-Asn (CDR_{1H}), Met-Ile-Asp-Pro-Ser-Asp-Ser-Glu-Thr-Gln-Tyr-Asn-Gln-Met-Phe-Lys-Asp (CDR_{2H}) and Gly-Gly-Ala-Ser-Gly-Asp-Trp-Tyr-Phe-Asp-Val (CDR_{3H}) according to SEQ. ID NO:10 and 11.

Especially preferred are recombinant antibodies comprising a heavy chain variable domain of formula I, wherein the polypeptide residues of the flamework regions FR_1, FR_2, FR_3 and FR_4 are those preferably occurring in mammalian, especially murine or human, antibodies.

In a first embodiment of the invention, most preferred are recombinant antibodies with a heavy chain variable domain comprising a polypeptide of the amino acid sequence 2 to 120, of SEQ ID NO:4 and 5, wherein optionally one or more, e.g. 1, 2, 3 or 4, single amino acids within the amino acid sequences 2 to 31 (FR_1), 37 to 50 (FR_2), 68 to 99 (FR_3), and/or 110 to 120 (FR_4), are replaced by other amino acids or deleted, and wherein the amino acid Cys may be in the oxidized state forming S—S-bridges, in particular the recombinant antibodies with a heavy chain variable domain comprising a polypeptide of the amino acid sequence 2 to 120 of SEQ ID NO:4 and 5, wherein the amino acid Cys may be in the oxidized state forming S—S-bridges.

In a second embodiment of the invention, most preferred are recombinant antibodies wherein the heavy chain variable domain comprises a polypeptide of the amino acid sequence 2 to 121, of SEQ ID NO:10 and 11, wherein optionally one or more, e.g. 1, 2, 3 or 4, single amino acids within the amino acid sequences 2 to 31 (FR_1), 37 to 50 (FR_2), 68 to 99 (FR_3), and/or 111 to 121 (FR_4), are replaced by other amino acids or deleted, and wherein the amino acid Cys may be in the oxidized state forming S—S-bridges, in particular the recombinant antibodies with a heavy chain variable domain comprising a polypeptide of the amino acid sequence 2 to 121 of SEQ ID NO:10 and 11, wherein the amino acid Cys may be in the oxidized state forming S—S-bridges.

For example, a hydrophobic amino acid within the flamework regions may be replaced by another amino acid, preferably also a hydrophobic amino acid, e.g. a homologous amino acid, replaced by two amino acids, or deleted. Likewise, a hydrophilic amino acid within the flamework region may be replaced by another amino acid, two amino acids or deleted, whereby replacing amino acids preferably maintain the hydrogen bond structure of the corresponding flamework region.

Likewise preferred recombinant antibodies of the invention are those wherein the light chain variable domain comprises a polypeptide of the formula

$$FR_6\text{-}CDR_{1L}\text{-}FR_7\text{-}CDR_{2L}\text{-}FR_8\text{-}CDR_{3L}\text{-}FR_9 \quad (II)$$

wherein FR_6 is a polypeptide residue comprising naturally occurring amino acids, preferably 19–25, especially 19–23 naturally occurring amino acids, FR_7 is a polypeptide residue comprising 13–17 naturally occurring amino acids, FR_8 is a polypeptide residue comprising 30–34 naturally occurring amino acids, FR_9 is a polypeptide residue comprising naturally occurring amino acids, particularly 7–11 naturally occurring amino acids, and CDR_{1L} is a polypeptide residue of the amino acid sequence 159 to 169 of SEQ ID NO:4 and 5, CDR_{2L} is a polypeptide residue of the amino acid sequence 185 to 191 of SEQ ID NO:4 and 5, and CDR_{3L} is a polypeptide residue of the amino acid sequence 224 to 232 of SEQ ID NO:4 and 5, or CDR_{1L} is a polypeptide residue of the amino acid sequence 160 to 170 of SEQ ID NO:10 and 11, CDR_{2L} is a polypeptide residue of the amino acid sequence 186 to 192 of SEQ ID NO:10 and 11, and CDR_{3L} is a polypeptide residue of the amino acid sequence 225 to 232 of SEQ ID NO:10 and 11, and wherein the amino acid Cys may be in the oxidized state forming S—S-bridges. These particular complementarity determining regions are Lys-Ala-Ser-Gln-Asp-Val-Tyr-Asn-Ala-Val-Ala (CDR_{1L}), Ser-Ala-Ser-Ser-Arg-Tyr-Thr (CDR_{2L}), and Gln-Gln-His-Phe-Arg-Thr-Pro-Phe-Thr (CDR_{3L}) according to SEQ ID No:4 and 5, or Lys-Ala-Ser-Gln-Asp-Ile-Lys-Lys-Tyr-Ile-Ala (CDR_{1L}), Tyr-Thr-Ser-Val-Leu-Gln-Pro (CDR_{2L}) and Leu-His-Tyr-Asp-Tyr-Leu-Tyr-Thr (CDR_{3L}) according to SEQ ID NO:10 and 11.

Especially preferred are recombinant antibodies comprising a light chain variable domain of formula II, wherein the polypeptide residues of the framework regions FR_5, FR_6, FR_7 and FR_8 are those preferably occurring in mammalian, especially murine or human, antibodies.

In one embodiment of the invention, most preferred are recombinant antibodies wherein the light chain variable domain comprises a polypeptide of the amino acid sequence 136 to 241 of SEQ ID NO:4 and 5, wherein optionally one or more, e.g. 1, 2, 3 or 4, single amino acids within the amino acid sequences 136 to 158 (FR_6), 170 to 184 (FR_7), 192 to 223 (FR_8), and/or 233 to 241 (FR_9) are replaced by other amino acids or deleted, and wherein the amino acid Cys may be in the oxidized state forming S—S-bridges, in particular the recombinant antibodies with a light chain variable domain comprising a polypeptide of the amino acid sequence 136 to 241 of SEQ ID NO:4 and 5, wherein the amino acid Cys may be in the oxidized state forming S—S-bridges.

In a second embodiment of the invention, most preferred are recombinant antibodies wherein the light chain variable domain comprises a polypeptide of the amino acid sequence 137 to 241 of SEQ ID NO:10 and 11, wherein optionally one or more, e.g. 1, 2, 3 or 4 single amino acids within the amino acid sequences 137 to 159 (FR_6), 171 to 185 (FR_7), 193 to 224 (FR_8), and/or 233 to 241 (FR_9) are replaced by other amino acids or deleted, and wherein the amino acid Cys may be in the oxidized state forming S—S-bridges, in particular the recombinant antibody wherein the light chain variable domain comprises a polypeptide of the amino acid sequence 137 to 241 of SEQ ID NO:10 and 11, wherein the amino acid Cys may be in the oxidized state forming S—S-bridges.

For example, amino acids within the framework regions may be replaced by other amino acids or deleted as detailed above for the heavy chain.

Especially preferred is a single-chain recombinant antibody wherein the heavy chain variable domain and the light chain variable domain are linked by way of a spacer group consisting of 10 to 30, e.g. around 15, amino acids, in particular a single-chain recombinant antibody comprising a polypeptide of the formula

$$FR_1\text{-}CDR_{1H}\text{-}FR_2\text{-}CDR_{2H}\text{-}FR_3\text{-}CDR_{3H}\text{-}FR_4\text{-}Sp\text{-}FR_6\text{-}CDR_{1L}\text{-}FR_7\text{-}CDR_{2L}\text{-}FR_8\text{-}CDR_{3L}\text{-}FR_9 \quad (III),$$

wherein FR_1, CDR_{1H}, FR_2, CDR_{2H}, FR_3, CDR_{3H}, FR_4, FR_6, CDR_{1L}, FR_7, CDR_{2L}, FR_8, CDR_{3L} and FR_9 have the meanings as mentioned before and Sp is a peptide spacer consisting of about 10 to 30, e.g. around 15, amino acids; and wherein the heavy chain or the light chain variable domain is further connected to an effector molecule, e.g. an enzyme, such as phosphatase, particularly alkaline phosphatase, or a toxin, such as Pseudomonas exotoxin, or a variant thereof. Preferably, the effector molecule is connected to the light chain variable domain, optionally via a peptide spacer consisting of one or more, e.g. 1–10 amino acids.

These fusion proteins comprising a single-chain recombinant antibody and an effector molecule optionally comprise another peptide, e.g. a peptide facilitating purification, in particular a peptide being an epitope against which an antibody is available, such as the FLAG peptide. Purification, e.g. by means of affinity chromatography, of a fusion protein comprising such a peptide is advantageous e.g. in that it may be faster, more specific and/or gentler. The peptide may be placed at the N-terminus of the fusion protein, in between the recombinant antibody and the effector molecule, or at the C-terminus of the fusion protein. Preferably, it is located at the N-terminus or at the C-terminus, in particular at the N-terminus. Preferably, these constructs also contain a cleavage site, so that the fusion protein can be liberated therefrom, either by enzymatic cleavage, e.g. by enterokinase or by Factor Xa, or by the chemical methods known in the art. Furthermore these constructs may comprise a peptide spacer consisting of one or more, e.g. 1 to 10, in particular about 2 amino acids, said spacer facilitating the linkage of the above-mentioned peptide and/or the cleavage site to the recombinant antibody. The cleavage site is placed in such a way that the fusion protein comprising the recombinant antibody and the effector molecule can be easily liberated, if desired, preferably in vitro. For example, in the protein construct comprising the fusion protein designated Fv(FRP5)-ETA (cf. SEQ. ID NO:13 and 14), the FLAG peptide and an enterokinase cleavage site are linked to a spacer and placed in front of the Fv heavy chain/light chain variable domain and exotoxin A fusion protein. If desired, the FLAG peptide can be cleaved off by enterokinase, preferably after affinity purification of the protein, yielding a fusion protein comprising the single-chain antibody Fv(FRP5) and exotoxin A.

Most preferred is a single-chain recombinant antibody wherein the heavy chain variable domain and the light chain variable domain are derived from a mouse monoclonal antibody directed to the extracellular domain of the growth factor receptor c-erbB-2, e.g. derived from the mouse monoclonal antibodies FRP5, FSP16, FWP51 or FSP77, particularly from the mouse monoclonal antibodies FRP5 or FWP51. Likewise preferred is a single-chain recombinant antibody wherein the spacer group linking the light chain and the heavy chain variable domains is a polypeptide comprising about 15 amino acids selected from glycine and serine, in particular wherein the spacer group is the 15 amino acid polypeptide consisting of three repetitive subunits of Gly-Gly-Gly-Gly-Ser.

Especially preferred is a single-chain antibody comprising the heavy chain variable domain of a mouse monoclonal antibody selected from the group consisting of FRP5, FSP16, FWP51 and FSP77, the 15 amino acid spacer group consisting of three repetitive subunits of Gly-Gly-Gly-Gly-Ser, the light chain variable domain of a mouse monoclonal-antibody selected from the group consisting of FRP5, FSP16, FWP51 and FSP77 and an enzyme, for example a phosphatase such as the alkaline phosphatase phoA, or an exotoxin such as Pseudomonas exotoxin, or a variant thereof.

Particularly preferred is the particular single-chain recombinant antibody designated Fv(FRP5)-phoA comprising a polypeptide of the amino acid sequence 2 to 690 of SEQ ID NO:6 and 7.

Likewise preferred is a single-chain recombinant antibody comprising a peptide facilitating purification, a cleavage site and a particular single-chain recombinant antibody selected from the group consisting of Fv(FRP5)-ETA and Fv(FWP51)-ETA, in particular a single-chain recombinant antibody comprising a polypeptide selected from the group consisting of a polypeptide of the amino acid sequence -10 to 606 of SEQ. ID NO:13 and 14 and of a polypeptide of the amino acid sequence -10 to 606 of SEQ. ID NO:15 and 16, said protein being subjected to in vitro cleavage by enterokinase, if desired.

Particularly preferred is a single-chain recombinant antibody comprising a protein selected from the group consisting of a polypeptide of the amino acid sequence 2 to 606 of SEQ ID NO:13 and 14 and a polypeptide of the amino acid sequence 2 to 606 of SEQ ID NO:15 and 16.

The invention further concerns the mouse monoclonal antibodies directed to the extracellular domain of the growth factor receptor c-erbB-2 and designated FRP5, FSP16, FSP77, and FWP51, which are secreted by the hybridoma cell lines FRP5, FSP16, FSP77, and FWP51, respectively. Most preferred are the mouse monoclonal antibodies designated FRP5 and FWP51.

The invention further concerns a method of manufacture of the recombinant antibodies and of the mouse monoclonal antibodies of the invention. The antibodies are prepared by processes that are known per se, characterized in that host cells or hybridoma cells as defined further below producing such antibodies are multiplied in vitro or in vivo and, when required, the obtained antibodies are isolated. For example, the recombinant antibodies of the invention can be prepared by recombinant DNA techniques comprising culturing a transformed host under conditions which allow expression thereof and isolating said antibody.

More specifically, the present invention also relates to a process for the production of a protein of the invention selected from the group consisting of a heavy chain murine variable domain, a light chain murine variable domain, a heavy chain murine variable domain and a light chain murine variable domain, a single-chain recombinant antibody, a fusion protein, and a fusion protein optionally comprising a peptide facilitating purification, a cleavage site and a peptide spacer comprising culturing a host, e.g. *E. coli*, which has been transformed with a hybrid vector comprising an expression cassette comprising a promoter and a DNA coding for said protein which DNA is controlled by said promoter, and isolating said protein.

In particular, the present invention relates to a process for the production of a protein of the invention selected from the group consisting of a heavy chain murine variable domain, a light chain murine variable domain, a heavy chain murine variable domain and a light chain murine variable domain, a single-chain recombinant antibody, and a fusion protein optionally comprising a peptide facilitating purification, a cleavage site and a peptide spacer comprising culturing a host, e.g. *E. coli*, which has been transformed with a hybrid vector comprising an expression cassette comprising a promoter operably linked to a first DNA sequence encoding a signal peptide linked in the proper reading frame to a second DNA sequence encoding said protein, and isolating said protein.

Multiplication of hybridoma cells or mammalian host cells in vitro is carried out in suitable culture media, which are the customary standard culture media, for example Dulbecco's Modified Eagle Medium (DMEM) or RPMI 1640 medium, optionally replenished by a mammalian serum, e.g. fetal calf serum, or trace elements and growth sustaining supplements, e.g feeder cells such as normal mouse peritoneal exudate cells, spleen cells, bone marrow macrophages, 2-aminoethanol, insulin, transferrin, low density lipoprotein, oleic acid, or the like. Multiplication of host cells which are bacterial cells or yeast cells is likewise carried out in suitable culture media known in the art, for example for bacteria in medium LB, NZCYM, NZYM, NZM, Terrific Broth, SOB, SOC, 2xYT, or M9 Minimal Medium, and for yeast in medium YPD, YEPD, Minimal Medium, or Complete Minimal Dropout Medium.

In vitro production provides relatively pure antibody preparations and allows scale-up to give large amounts of the desired antibodies. Techniques for bacterial cell, yeast or mammalian cell cultivation are known in the art and include homogeneous suspension culture, e.g. in an airlift reactor or in a continuous stirrer reactor, or immobilized or entrapper cell culture, e.g. in hollow fibres, microcapsules, on agarose microbeads or ceramic cartridges.

Large quantities of the desired antibodies can also be obtained by multiplying mammalian cells in vivo. For this purpose, hybridoma cells producing the desired antibodies are injected into histocompatible mammals to cause growth of antibody-producing tumors. Optionally, the animals are primed with a hydrocarbon, especially mineral oils such as pristane (tetramethyl-pentadecane), prior to the injection. After one to three weeks, the antibodies are isolated from the body fluids of those mammals. For example, hybridoma cells obtained by fusion of suitable myeloma cells with antibody-producing spleen cells from Balb/c mice, or transfected cells derived from hybridoma cell line Sp2/0 that produce the desired antibodies are injected intraperitoneally into Balb/c mice optionally pre-treated with pristane, and, after one to two weeks, ascitic fluid is taken from the animals.

The cell culture supernatants are screened for the desired antibodies, preferentially by immunofluorescent staining of cells expressing c-erbB-2, by immunoblotting, by an enzyme immunoassay, e.g. a sandwich assay or a dot-assay, or a radioimmunoassay.

For isolation of the antibodies, the immunoglobulins in the culture supernatants or in the ascitic fluid may be concentrated, e.g. by precipitation with ammonium sulphate, dialysis against hygroscopic material such as polyethylene glycol, filtration through selective membranes, or the like. If necessary and/or desired, the antibodies are purified by the customary chromatography methods, for example gel filtration, ion-exchange chromatography, chromatography over DEAE-cellulose and/or (immuno-)affinity chromatography, e.g. affinity chromatography with c-erbB-2 protein or with Protein-A.

The invention further concerns hybridoma cells secreting the monoclonal antibodies of the invention, in particular the

hybridoma cell lines FRP5, FSP16, FSP77, and FWP51 deposited under the Budapest Treaty on Nov. 21, 1990 at the European Collection of Animal Cell Cultures (ECACC) in Porton Down, Salibury, UK, under the accession numbers 90112115, 90112116, 90112117, and 90112118, respectively. Most preferred is the hybridoma cell line designated FRP5, ECACC number 90112115 or the hybridoma cell line designated FWP51, ECACC number 90112118. The preferred hybridoma cells of the invention are genetically stable, secrete monoclonal antibodies of the invention of the desired specificity and can be activated from deep-frozen cultures by thawing and recloning.

The invention also concerns a process for the preparation of a hybridoma cell line secreting monoclonal antibodies directed to the extracellular domain of the growth factor receptor c-erbB-2, characterized in that a suitable mammal, for example a Balb/c mouse, is immunized with purified c-erbB-2 protein, an antigenic carrier containing purified c-erbB-2 or with cells bearing growth factor receptor c-erbB-2, antibody-producing cells of the immunized mammal are fused with cells of a suitable myeloma cell line, the hybrid cells obtained in the fusion are cloned, and cell clones secreting the desired antibodies are selected. For example spleen cells of Balb/c mice immunized with cells bearing c-erbB-2 are fused with cells of the myeloma cell line PAI or the myeloma cell line Sp2/0-Ag14, the obtained hybrid cells are screened for secretion of the desired antibodies, and positive hybridoma cells are cloned.

Preferred is a process for the preparation of a hybridoma cell line, characterized in that Balb/c mice are immunized by injecting subcutaneously and/or intraperitoneally between 10^7 and 10^8 cells of the human breast tumor cell line SKBR3 containing a suitable adjuvant several times, e.g. four to six times, over several months, e.g. between two and four months, and spleen cells from the immunized mice are taken two to four days after the last injection and fused with cells of the myeloma cell line PAI in the presence of a fusion promoter, preferably polyethylene glycol. Preferably the myeloma cells are fused with a three- to twentyfold excess of spleen cells from the immunized mice in a solution containing about 30% to about 50% polyethylene glycol of a molecular weight around 4000. After the fusion the cells are expanded in suitable culture media as described hereinbefore, supplemented with a selection medium, for example HAT medium, at regular intervals in order to prevent normal myeloma cells from overgrowing the desired hybridoma cells.

The invention also concerns recombinant DNAs comprising an insert coding for a heavy chain murine variable domain and/or for a light chain murine variable domain of antibodies directed to the extracellular domain of the growth factor receptor c-erbB-2 as described hereinbefore. By definition such DNAs comprise coding single stranded DNAs, double stranded DNAs consisting of said coding DNAs and of complementary DNAs thereto, or these complementary (single stranded) DNAs themselves.

Furthermore, DNA encoding a heavy chain murine variable domain and/or for a light chain murine variable domain of antibodies directed to the extracellular domain of the growth factor receptor c-erbB-2 can be enzymatically or chemically synthezised DNA having the authentic DNA sequence coding for a heavy chain murine variable domain and/or for the light chain murine variable domain, or a mutant therof. A mutant of the authentic DNA is a DNA encoding a heavy chain murine variable domain and/or a light chain murine variable domain of the above-mentioned antibodies in which one or more amino acids are deleted or exchanged with one or more other amino acids. Preferably said modification(s) are outside the CDRs of the heavy chain murine variable domain and/or of the light chain murine variable domain of the antibody. Such a mutant DNA is also intended to be a silent mutant wherein one or more nucleotides are replaced by other nucleotides with the new codons coding for the same amino acid(s). Such a mutant sequence is also a degenerated sequence. Degenerated sequences are degenerated within the meaning of the genetic code in that an unlimited number of nucleotides are replaced by other nucleotides without resulting in a change of the amino acid sequence originally encoded. Such degenerated sequences may be useful due to their different restriction sites and/or frequency of particular codons which are preferred by the specific host, particularly *E. coli*, to obtain an optimal expression of the heavy chain murine variable domain and/or a light chain murine variable domain.

The term mutant is intended to include a DNA mutant obtained by in vitro mutagenesis of the authentic DNA according to methods known in the art.

The invention relates to a recombinant DNA comprising an insert coding for a heavy chain murine variable domain of a monoclonal antibody selected from the group consisting of the antibodies FRP5, FSP16, FSP77 and FWP51, or coding for an amino acid sequence homologous to said heavy chain variable domain.

In particular, the invention concerns a recombinant DNA comprising an insert coding for a heavy chain murine variable domain, which originates from genomic DNA or mRNA of the hybridoma cell lines FRP5, FSP16, FSP77 or FWP51, or which is homologous to genomic DNA of said cell lines and codes for an amino acid sequence homologous to the heavy chain variable domain of monoclonal antibodies FRP5, FSP16, FSP77 or FWP51. Especially preferred is a recombinant DNA comprising an insert coding for a heavy chain murine variable domain, which originates from genomic DNA or mRNA of the hybridoma cell line FRP5, or which is homologous to genomic DNA of said cell line and codes for an amino acid sequence homologous to the heavy chain variable domain of monoclonal antibody FRP5; or a recombinant DNA comprising an insert coding for a heavy chain murine variable domain, which originates from genomic DNA or mRNA of the hybridoma cell line FWP51, or which is homologous to genomic DNA of said cell line and codes for an amino acid sequence homologous to the heavy chain variable domain of monoclonal antibody FWP51

Preferred is a recombinant DNA comprising an insert coding for the polypeptide of formula I, wherein FR_1, FR_2, FR_3, FR_4, CDR_{1H}, CDR_{2H}, and CDR_{3H} have the meanings as mentioned hereinbefore, optionally further containing introns. Especially preferred is a recombinant DNA coding for the polypeptide of formula I comprising inserts coding for murine or human framework regions FR_1, FR_2, FR_3 and FR_4, and inserts coding for complementarity determining regions of the DNA sequence 99 to 113 (CDR_{1H}), the DNA sequence 156 to 206 (CDR_{2H}), and the DNA sequence 303 to 332 (CDR_{3H}) of SEQ ID NO:4 and 5 or coding for complementarity determining regions of the DNA sequence 99 to 113 (CDR_{1H}), the DNA sequence 156 to 206 (CDR_{2H}), and the DNA sequence 303 to 335 (CDR_{3H}) of SEQ ID NO:10 and 11. Most preferred is a DNA comprising an insert of the DNA sequence 9 to 365 of SEQ ID NO:4 and 5, wherein optionally one or more, e.g. 1 to 10, nucleotides are replaced by other nucleotides, in particular a DNA comprising an insert of the DNA sequence 9 to 365 of SEQ ID NO:4 and 5. Likewise preferred is a DNA comprising an insert of

the DNA sequence 9 to 368 of SEQ ID NO:10 and 11, wherein optionally one or more, e.g. 1 to 10, nucleotides are replaced by other nucleotides, in particular a DNA comprising an insert of the DNA sequence 9 to 368 of SEQ ID NO:10 and 11.

In a DNA wherein nucleotides of the sequence given in SEQ ID NO:4 and 5, or in a DNA wherein nucleotides of the sequence given in SEQ ID NO:10 and 11, are replaced by other nucleotides, such replacement is preferred when it does not alter the amino acid sequence of the complementarity determining regions (CDRs) coded for. This means that such replacement of nucleotides may occur in the inserts coding for the framework regions (FRs) or in a position where it does not alter the amino acid coded for due to the degeneracy of the triplet codons.

Likewise the invention relates to a recombinant DNA comprising an insert coding for a light chain murine variable domain of a monoclonal antibody selected from the group consisting of the antibodies FRP5, FSP16, FSP77 and FWP51, or coding for an amino acid sequence homologous to said light chain variable domain.

More specifically, the invention concerns a recombinant DNA comprising an insert coding for a light chain murine variable domain, which originates from genomic DNA or mRNA of the hybridoma cell lines FRP5, FSP16, FSP77 or FWP51, or which is homologous to genomic DNA of said cell lines and codes for an amino acid sequence homologous to the light chain variable domain of monoclonal antibodies FRP5, FSP16, FSP77 or FWP51. Particularly preferred is a recombinant DNA comprising an insert coding for a light chain murine variable domain, which originates from genomic DNA or mRNA of the hybridoma cell line FRP5, or which is homologous to genomic DNA of said cell line and codes for an amino acid sequence homologous to the light chain variable domain of monoclonal antibody FRP5, or a recombinant DNA comprising an insert coding for a light chain murine variable domain, which originates from genomic DNA or mRNA of the hybridoma cell line FwP51, or which is homologous to genomic DNA of said cell line and codes for an amino acid sequence homologous to the light chain variable domain of monoclonal antibody FWP51.

Preferred is a recombinant DNA comprising an insert coding for the polypeptide of formula II, wherein FR_5, FR_6, FR_7, FR_8, CDR_{1L}, CDR_{2L}, and CDR_{3L} have the meanings as mentioned hereinbefore, optionally further containing introns. Especially preferred is a recombinant DNA coding for the polypeptide of formula II comprising inserts coding for murine or human framework regions FR_5, FR_6, FR_7 and FR_8, and inserts coding for complementarity determining regions of the DNA sequence 480 to 512 (CDR_{1L}), the DNA sequence 558 to 578 (CDR_{2L}), and the DNA sequence 675 to 701 (CDR_{3L}) of SEQ ID NO:4 and 5, or coding for complementarity determining regions of the DNA sequence 483 to 515 (CDR_{1L}), the DNA sequence 561 to 581 (CDR_{2L}), and the DNA sequence 678 to 701 (CDR_{3L}) of SEQ ID NO:10 and 11.

Most preferred is a DNA comprising an insert of the DNA sequence 411 to 728 of SEQ ID NO:4 and 5, wherein optionally one or more, e.g. 1 to 10, nucleotides are replaced by other nucleotides, in particular a DNA comprising an insert of the DNA sequence 411 to 728 of SEQ ID NO:4 and 5. Likewise preferred is a DNA comprising an insert of the DNA sequence 414 to 728 of SEQ ID NO:10 and 11, wherein optionally one or more, e.g. 1 to 10, nucleotides are replaced by other nucleotides, in particular a DNA comprising an insert of the DNA sequence 414 to 728 of SEQ ID NO:10 and 11. In a DNA wherein nucleotides of the sequence given in SEQ ID NO:4 and 5, or in a DNA wherein nucleotides of the sequence given in SEQ ID NO:10 and 11, are replaced by other nucleotides, such replacement is preferred when it does not alter the amino acid sequence of the complementarity determining regions (CDRs) coded for, as is described above for DNA coding for the heavy chain variable domain.

For the assembly of complete tetrameric immunoglobulin molecules and the expression of chimeric antibodies, the recombinant DNA inserts coding for heavy and light chain variable domains are fused with the corresponding DNAs coding for heavy and light chain constant domains, then transferred into appropriate host cells, for example after incorporation into hybrid vectors.

The invention therefore also concerns recombinant DNAs comprising an insert coding for a heavy chain murine variable domain of an antibody directed to the extracellular domain of c-erbB-2 fused to a human constant domain γ, for example γ1, γ2, γ3 or γ4, preferably γ1 or γ4. Likewise the invention concerns recombinant DNAs comprising an insert coding for a light chain murine variable domain of an antibody directed to the extracellular domain of c-erbB-2 fused to a human constant domain κ or λ, preferably κ.

The invention especially concerns recombinant DNAs coding for a single-chain recombinant antibody as defined hereinbefore, e.g. recombinant DNA wherein the heavy chain variable domain and the light chain variable domain are linked by way of a DNA insert coding for a spacer group, in particular a recombinant DNA coding for a protein of the formula III, wherein FR_1, FR_2, FR_3, FR_4, FR_6, FR_7, FR_8, FR_9, SP, CDR_{1H}, CDR_{2H}, CDR_{3H}, CDR_{1H}, CDR_{2L} and CDR_{3L} have the meanings given above, optionally comprising further DNA coding for an effector molecule and/or signal sequences facilitating the processing of the antibody in the host cell. In particular the invention concerns a DNA comprising an insert of the DNA sequence 9–728 of SEQ ID NO:4 and 5, wherein optionally one or more, e.g. 1 to 10, nucleotides are replaced by other nucleotides, especially a DNA comprising an insert of the DNA sequence 9 to 728 of SEQ ID NO:4 and 5. Furthermore the invention relates to a DNA comprising an insert of the DNA sequence 9–728 of SEQ ID NO:10 and 11 wherein optionally one or more, e.g. 1 to 10, nucleotides are replaced by other nucleotides, especially a DNA comprising an insert of the DNA sequence 9 to 728 of SEQ ID NO:10 and 11.

In another embodiment the invention pertains to recombinant DNAs coding for a recombinant DNA wherein the heavy chain variable domain and the light chain variable domain are linked by way of a DNA insert coding for a spacer group, optionally comprising a signal sequence facilitating the processing of the antibody in the host cell and/or a DNA coding for a peptide facilitating the purification of the antibody and/or a DNA coding for a cleavage site and/or a DNA coding for a peptide spacer and/or a DNA coding for an effector molecule.

The DNA coding for an effector molecule is intended to be a DNA coding for the above-mentioned effector molecules, particularly a DNA coding for alkaline phosphatase or Pseudomonas exotoxin A. The DNA encoding such an effector molecule has the sequence of a naturally occurring enzyme or toxin encoding DNA, or a mutant therof, and can be prepared by methods well known in the art. A mutant of the naturally occurring DNA encoding e.g. alkaline phosphatase or Pseudomonas exotoxin A, or a variant thereof can be obtained e.g. analogously to the methods described above.

Most preferred is a DNA comprising an insert of the DNA sequence 23 to 814 of SEQ ID NO:6 and 7, of the DNA

sequence 86 to 2155 of SEQ ID NO:5 or of the DNA sequence 23 to 2155 of SEQ ID NO:6 and 7, wherein optionally one or more, e.g. 1 to 10, nucleotides are replaced by other nucleotides, in particular a DNA comprising an insert of the DNA sequence 23 to 2155 of SEQ ID NO:6 and 7.

Equally preferred is a DNA comprising an insert of the DNA sequence 1 to 1911 of SEQ ID NO:13 and 14, of the DNA sequence 64 to 1911 of SEQ ID NO:13 and 14, or of the DNA sequence 97 to 1911 of SEQ ID NO:13 and 14, wherein optionally one or more, e.g. 1 to 10, nucleotides are replaced by other nucleotides, in particular a DNA comprising an insert of the DNA sequence 1 to 1911 of SEQ IDs NO:13 and 14; or a DNA comprising an insert of the DNA sequence 1 to 1911 of SEQ ID NO:15 and 16, of the DNA sequence 64 to 1911 of SEQ ID NO:15 and 16, of the DNA sequence 96 to 1911 of SEQ ID NO:15 and 16, or of the DNA sequence 97 to 1911 of SEQ ID NO:15 and 16, wherein optionally one or more, e.g. 1 to 10, nucleotides are replaced by other nucleotides, in particular a DNA comprising an insert of the DNA sequence 1 to 1911 of SEQ ID NO:15 and 16.

Furthermore the invention concerns a recombinant DNA which is a hybrid vector comprising an insert coding for the variable domain of a murine heavy chain as described hereinbefore and/or an insert coding for the variable domain of a murine light chain as described hereinbefore, an origin of replication or an autonomously replicating sequence, one or more dominant marker sequences and, optionally, expression control sequences, signal sequences and additional restriction sites.

In a first embodiment the hybrid vector according to the invention comprises an expression cassette comprising a promoter and a DNA coding for a protein of the invention selected from the group consisting of a heavy chain murine variable domain, a light chain murine variable domain, a heavy chain murine variable domain and a light chain murine variable domain, a single-chain recombinant antibody, a fusion protein, and a fusion protein optionally comprising a peptide facilitating purification, a cleavage site and a peptide spacer, which DNA is controlled by said promoter, and isolating said protein.

In a second embodiment, the hybrid vector according to the invention comprises an expression cassette comprising a promoter operably linked to a first DNA sequence encoding a signal peptide linked in the proper reading frame to a second DNA sequence encoding a protein of the invention selected from the group consisting of a heavy chain murine variable domain, a light chain murine variable domain, a heavy chain murine variable domain and a light chain murine variable domain, a single-chain recombinant antibody, and a fusion protein optionally comprising a peptide facilitating purification, a cleavage site and a peptide spacer.

Vectors typically perform two functions in collaboration with compatible host cells. One function is to facilitate the cloning of the nucleic acid that encodes the immunoglobulin variable domains, i.e. to produce usable quantities of the nucleic acid (cloning vectors). The other function is to provide for replication and expression of the recombinant gene constructs in a suitable host, either by maintenance as an extrachromosomal element or by integration into the host chromosome (expression vectors). A cloning vector comprises the recombinant gene constructs as described above, an origin of replication or an autonomously replicating sequence, dominant marker sequences and, optionally, signal sequences and additional restriction sites. An expression vector additionally comprises expression control sequences essential for the transcription and translation of the recombinant genes.

An origin of replication or an autonomously replicating sequence is provided either by construction of the vector to include an exogeneous origin such as derived from Simian virus 40 (SV 40) or another viral source, or by the host cell chromosomal mechanisms.

The markers allow for selection of host cells which contain the vector. Selection markers include genes which confer resistance to heavy metals such as copper or to antibiotics such as geneticin (G-418) or hygromycin, or genes which complement a genetic lesion of the host cell such as the absence of thymidin kinase, hypoxanthine phosphoryl transferase, dihydrofolate reductase or the like.

Signal sequences may be, for example, presequences or secretory leaders directing the secretion of the recombinant antibody, splice signals, or the like. Examples for signal sequences directing the secretion of the recombinant antibody are sequences derived from the ompA gene, the pelB (pectate lyase) gene or the phoA gene.

As expression control sequences, the vector DNA comprises a promoter, sequences necessary for the initiation and termination of transcription and for stabilizing the mRNA and, optionally, enhancers and further regulatory sequences.

A wide variety of promoting sequences may be employed, depending on the nature of the host cell. Promoters that are strong and at the same time well regulated are the most useful. Sequences for the initiation of translation are for example Shine-Dalgarno sequences. Sequences necessary for the initiation and termination of transcription and for stabilizing the mRNA are commonly available from the noncoding 5'-regions and 3'-regions, respectively, of viral or eukaryotic cDNAs, e.g. from the expression host. Enhancers are transcription-stimulating DNA sequences of viral origin, e.g. derived from Simian virus, polyoma virus, bovine papilloma virus or Moloney sarcoma virus, or of genomic, especially murine, origin.

The various DNA segments of the vector DNA are operationally linked, i.e. they are contiguous and placed into a functional relationship with each other. Examples of vectors which are suitable for replication and expression in an *E. coli* strain are bacteriophages, for example derivatives of λ bacteriophages, or plasmids, such as, in particular, the plasmid ColE1 and its derivatives, for example pMB9, pSF2124, pBR317 or pBR322 and plasmids derived from pBR322, such as pUC9, pUCK0, pHRi148 and pLc24. Suitable vectors contain a complete replicon, a marker gene, recognition sequences for restriction endonucleases, so that the foreign DNA and, if appropriate, the expression control sequence can be inserted at these sites, and optionally signal sequences and enhancers.

Microbial promoters are, for example, the strong leftward promoter P_L of bacteriophage λ which is controlled by a temperature sensitive repressor. Also suitable are *E. coli* promoters such as the lac (lactose) promoter regulated by the lac repressor and induced by isopropyl-β-D-thiogalactoside, the trp (tryptophan) promoter regulated by the trp repressor and induced e.g. by tryptophan starvation, and the tac (hybrid trp-lac promoter) regulated by the lac repressor.

Vectors which are suitable for replication and expression in yeast contain a yeast replication start and a selective genetic marker for yeast. One group of such vectors includes so-called ars sequences (autonomous replication sequences) as origin of replication. These vectors are retained extrachromosomally within the yeast cell after the transformation and are replicated autonomously. Furthermore, vectors which contain all or part of the 2μ (2 mikron) plasmid DNA from *Saccharomyces cerevisiae* can be used.

Such vectors will get integrated by recombination into 2μ plasmids already existing within the cell, or replicate autonomously. 2μ sequences are particularly suitable when high transformation frequency and high copy numbers are to be achieved.

Expression control sequences which are suitable for expression in yeast are, for example, those of highly expressed yeast genes. Thus, the promoters for the TRP1 gene, the ADHI or ADHII gene, acid phosphatase (PHO3 or PHO5) gene, isocytochrome gene or a promoter involved with the glycolytic pathway, such as the promoter of the enolase, glyceraldehyde-3-phosphate kinase (PGK), hexokinase, pyruvate decarboxylase, phosphofructokinase, glucose-6-phosphate isomerase, 3-phosphoglycerate mutase, pyruvate kinase, triosephosphate isomerase, phosphoglucose isomerase and glucokinase genes, can be used.

Vectors suitable for replication and expression in mammalian cells are preferably provided with promoting sequences derived from DNA of viral origin, e.g. from Simian virus 40 (SV40), Rous sarcoma virus (RSV), adenovirus 2, bovine papilloma virus (BPV), papovavirus BK mutant (BKV), or mouse or human cytomegalovirus (CMV). Alternatively, the vectors may comprise promoters from mammalian expression products, such as actin, collagen, myosin etc., or the native promoter and control sequences which are normally associated with the desired gene sequence, i.e. the immunoglobulin H-chain or L-chain promoter.

Preferred vectors are suitable for both procaryotic and eucaryotic hosts and are based on viral replication systems. Particularly preferred are vectors comprising Simian virus promoters, e.g. pSVgpt or pSVneo, further comprising an enhancer, e.g. an enhancer normally associated with the immunoglobulin gene sequences, in particular the mouse Ig H- or L-chain enhancer.

The recombinant DNA coding for a recombinant antibody of the invention can be prepared, for example, by culturing a transformed host cell and optionally isolating the prepared DNA.

In particular, such DNA can be prepared by a method comprising

a) preparing murine DNA coding for the variable heavy and/or light chain domains of the antibody with the desired specificity, e.g. by isolating the DNA from the genome of a suitable hybridoma cell line and selecting the desired DNA using DNA probes, or by isolating mRNA from a suitable hybridoma cell line and preparing cDNA coding for the variable heavy and/or light chain domains of the antibody with the desired specificity using oligonucleotide primers,

b) preparing DNA coding for the desired signal sequence and/or preparing DNA coding for an effector molecule, e.g. by isolating the desired DNA(s) from a suitable source, e.g. from a genomic library or a cDNA library using DNA probes,

c) synthesizing DNA coding for the desired spacer group by chemical methods,

d) constructing recombinant genes encoding the recombinant antibodies by incorporating the DNA of step a) and, optionally, b) and/or c) into appropriate hybrid vectors,

e) transferring the obtained hybrid vectors into a recipient host cell or retrieving the DNA coding for the recombinant genes and transferring the unlinked DNA into a recipient host cell,

f) selecting and culturing the transformed host cell, and

g) optionally isolating the desired DNA.

The DNA according to step a) of the process described above can be obtained by isolation of genomic DNA or by preparation of cDNA from isolated mRNA. Genomic DNA from hybridoma cells is isolated by methods known in the art which include steps for disruption of the cells, e.g. by lysis in presence of detergents like Triton™, extracting the DNA, e.g. by treatment with phenol and $CHCl_3$/isoamyl alcohol, and precipitation of DNA. The DNA is fragmented, conveniently by one or more restriction endonucleases, the resulting fragments are replicated on a suitable carrier, e.g. nitrocellulose membranes, and screened with a DNA probe for the presence of the DNA sequences coding for the polypeptide sequence of interest, in particular for the presence of the rearranged H- and L-chain Ig gene loci. By this procedure DNA fragments are found that contain inserts with heavy chain V, D and J regions and light chain V and J regions, respectively, together with a leader sequence and introns, if any. cDNA from hybridoma cells is likewise prepared by methods known in the art, e.g. by extracting total cellular RNA, isolating mRNA by a suitable chromatographic method, e.g. chromatography on oligo(dT)-cellulose, synthesizing cDNA with a mixture of deoxynucleotide triphosphates and reverse transcriptase in the presence of oligonucleotide primers complementary to suitable regions in the murine immunoglobulin heavy and light chain constant domain genes, and isolating the cDNA. As a tool simplifying DNA isolation, the desired genomic DNA or cDNA may be amplified using polymerase chain reaction (PCR) technology. PCR involves repeated rounds of extension from two primers specific for DNA regions at each end of the gene.

Preferably, cDNA transcripts of total mRNA from the suitable hybridoma cell line is treated in a heating/cooling cycle with Taq DNA polymerase in the presence of primers tailored to hybridize to Ig H- and L-chain variable domains, respectively.

Genomic DNA or cDNA according to step b) of the process described above is isolated from suitable bacterial or mammalian cells according to methods known in the art. Preferably, the methods as described under a) are used, substituting the corresponding source cells for the murine hybridoma cells and using DNA probes designed to hybridize with the desired signal sequences or the genes coding for the desired effector molecules. In bacteria wherein separation of mRNA from total RNA is not possible with olig(dT)-cellulose, cDNA is prepared from total RNA using corresponding oligonucleotide primers. The DNA isolation is simplified considerably by the PCR technology.

DNA according to step c) is prepared by conventional chemical and enzymatic methods, e.g. by chemical synthesis of oligonucleotides of between thirty and sixty bases with overlapping complementary sequences, hybridization of such oligonucleotides, and enzymatic ligation, optionally after filling-in of missing bases with suitable enzymes in the presence of the corresponding deoxynucleotide triphosphates.

The DNA probe for the mouse variable chain domains may be a synthetic DNA, a cDNA derived from mRNA coding for the desired immunoglobulin or a genomic DNA or DNA fragment of known nucleotide sequence. As probes for the detection and/or amplification of the rearranged Ig gene loci of the variable domains of L-/H-chains, DNA fragments of known nucleotide sequences of adjacent conserved variable or constant domains are selected which constitute the Ig loci of the L-/H-chain in the mammal from which the DNA is derived, e.g. Balb/c mice. The DNA probe is synthesized by chemical methods or isolated from suitable tissue of an appropriate mammal, e.g. Balb/c mouse liver, and purified by standard methods. If required, the probe

DNA is labelled, e.g. radioactively labelled by the well-known nick-translation technique, then hybridized with the DNA library in buffer and salt solutions containing adjuncts, e.g. calcium chelators, viscosity regulating compounds, proteins, non-specific DNA and the like, at temperatures favoring selective hybridization.

Once a fragment has been identified which contains the desired DNA sequence, this fragment may be further manipulated to remove nonessential DNA, modified at one or both termini, and treated to remove all or a portion of intervening sequences, or the like.

The joining of the various DNA fragments in order to produce recombinant genes encoding the recombinant antibodies is performed in accordance with conventional techniques, for example, by blunt- or staggered-end ligation, restriction enzyme digestion to provide for appropriate cohesive termini, filling-in cohesive ends as appropriate, alkaline phosphatase treatment to avoid undesirable joining, and ligation with appropriate ligases.

The transfer of the recombinant DNAs, e.g. the transfer of hybrid vectors, and the selection of transformed cells is described below.

Moreover, the invention relates to host cells transformed with the recombinant DNAs described above, namely host cells which are transformed with a DNA encoding the heavy chain and/or a DNA encoding the light chain of the desired recombinant antibody, in particular host cells transformed with a DNA encoding the preferred single-chain recombinant antibody.

More specifically, the invention concerns a host cell which has been transformed with a hybrid vector comprising an expression cassette comprising a promoter and a DNA coding for a protein of the invention selected from the group consisting of a heavy chain murine variable domain, a light chain murine variable domain, a heavy chain murine variable domain and a light chain murine variable domain, a single-chain recombinant antibody, a fusion protein, and a fusion protein further comprising a peptide facilitating purification, a cleavage site and a peptide spacer which DNA is controlled by said promoter.

Furthermore, the invention pertains to a host cell which has been transformed with a hybrid vector comprising an expression cassette comprising a promoter operably linked to a first DNA sequence encoding a signal peptide linked in the proper reading frame to a second DNA sequence encoding a protein of the invention selected from the group consisting of a heavy chain murine variable domain, a light chain murine variable domain, a heavy chain murine variable domain and a light chain murine variable domain, a single-chain recombinant antibody, a fusion protein, and a fusion protein further comprising a peptide facilitating purification, a cleavage site and a peptide spacer.

In particular, the present invention relates to a process for the production of a protein of the invention selected from the group consisting of a heavy chain murine variable domain, a light chain murine variable domain, a heavy chain murine variable domain and a light chain murine variable domain, a single-chain recombinant antibody, a fusion protein, and a fusion protein further comprising a peptide facilitating purification, a cleavage site and a peptide spacer comprising culturing a host, e.g. *E. coli*, which has been transformed with a hybrid vector comprising an expression cassette comprising a promoter operably linked to a first DNA sequence encoding a signal peptide linked in the proper reading frame to a second DNA sequence encoding said protein, and isolating said protein. The host cells of the present invention have to be capable of culture in vitro.

Suitable host cells are of procaryotic or of eucaryotic origin and are, for example, bacterial cells, e.g. *E. coli*, yeasts, e.g. *Saccharomyces cerevisiae*, or mammalian cells. For the preparation of functional chimeric human/mouse antibodies the host cells have to be of higher eucaryotic origin to provide a suitable environment for the production of activ. antibodies, since the biosynthesis of functional tetrameric antibody molecules requires correct nascent polypeptide chain folding, glycosylation, and assembly.

Examples of suitable hosts are microorganisms which are devoid of or poor in restriction enzymes or modification enzymes, such as bacteria, in particular strains of *Escherichia coli*, for example *E. coli* X1776, *E. coli* Y1090, *E. coli* HB 101, *E. coli* W3110, *E. coli* HB101/LM1035, *E. coli* JA 221, *E. coli* DH5α, *E. coli* K12, or *E. coli* CC118 strain, *Bacillus subtilis*, *Bacillus stearothermophilus*, Pseudomonas, Haemophilus, Streptococcus and others, and yeasts, for example *Saccharomyces cerevisiae* such as *S. cerevisiae* GRF 18. Further suitable host cells are cells of higher organisms, in particular established continuous human or animal cell lines, e.g. human embryonic lung fibroblasts L132, human malignant melanoma Bowes cells, HeLa cells, SV40 virus transformed kidney cells of African green monkey COS-7 or Chinese hamster ovary (CHO) cells, or cells of lymphoid origin, such as lymphoma, myeloma, hybridoma, trioma or quadroma cells, for example PAI, Sp2/0 or X63-Ag8.653 cells.

The above mentioned strains of *E. coli*, in particular *E. coli* CC118, are preferred as hosts.

The invention also concerns processes for the preparation of transformed host cells wherein suitable recipient host cells as described hereinbefore are transformed with a hybrid vector according to the invention, and the transformed cells are selected.

Transformation of microorganisms is carried out as described in the literature, for example for *S. cerevisiae* (A. Hinnen et al., Proc. Natl. Acad. Sci. USA 75: 1929, 1978), for *B. subtilis* (Anagnostopoulos et al., J. Bacteriol. 81: 741, 1961), and for *E. coli* (M. Mandel et al., J. Mol. Biol. 53: 159, 1970).

Accordingly, the transformation procedure of *E. coli* cells includes, for example, Ca^{2+} pretreatment of the cells so as to allow DNA uptake, and incubation with the hybrid vector. The subsequent selection of the transformed cells can be achieved, for example, by transferring the cells to a selective growth medium which allows separation of the transformed cells from the parent cells dependent on the nature of the marker sequence of the vector DNA. Preferably, a growth medium is used which does not allow growth of cells which do not contain the vector. The transformation of yeast comprises, for example, steps of enzymatic removal of the yeast cell wall by means of glucosidases, treatment of the obtained spheroplasts with the vector in the presence of polyethylene glycol and Ca^{2+} ions, and regeneration of the cell wall by embedding the spheroplasts into agar. Preferably, the regeneration agar is prepared in a way to allow regeneration and selection of the transformed cells as described above at the same time.

Transformation of cells of higher eucaryotic origin, such as mammalian cell lines, is preferably achieved by transfection. Transfection is carried out by conventional techniques, such as calcium phosphate precipitation, microinjection, protoplast fusion, electroporation, i.e. introduction of DNA by a short electrical pulse which transiently increases the permeability of the cell membrane, or in the presence of helper compounds such as diethylaminoethyl-dextran, dimethyl sulfoxide, glycerol or polyethylene glycol,

and the like. After the transfection procedure, transfected cells are identified and selected, for example, by cultivation in a selective medium chosen depending on the nature of the selection marker, for example standard culture media such as Dulbecco's modified Eagle medium (DMEM), minimum essential medium, RPMI 1640 medium and the like, containing e.g. the corresponding antibiotic.

The host cells are transformed with the recombinant L-chain gene construct alone, with the recombinant H-chain gene construct alone, with both, either sequentially or simultaneously, or by using a vector construct comprising both the L-chain and H-chain genes, for example a recombinant single-chain antibody gene construct as indicated hereinbefore.

Preferred are host cells transformed with a recombinant single-chain antibody gene construct comprising DNA coding for the heavy chain variable domain of an anti-c-erbB-2 antibody, DNA coding for a spacer group, DNA coding for the light chain variable domain of an anti-c-erbB-2 antibody and DNA coding for an effector molecule, in particular transfected with the preferred recombinant single-chain antibody gene construct as indicated hereinbefore. Further examples of host cells of the invention are cells transferred with similar recombinant plasmids which contain alternative orientations of the H- and L-chain gene constructs, and those incorporating additional DNA elements to facilitate high levels of expression of the recombinant antibodies.

The host cells of the invention are genetically stable, secrete recombinant antibodies of the invention of constant specificity and can be activated from deep-frozen cultures by thawing and recloning.

The transformed host cells are cultured by methods known in the art in a liquid medium containing assimilable sources of carbon, e.g. carbohydrates such as glucose or lactose, nitrogen, e.g. amino acids, peptides, proteins or their degradation products such as peptones, ammonium salts or the like, and inorganic salts, e.g. sulfates, phosphates and/or carbonates of sodium, potassium, magnesium and calcium. The medium furthermore contains, for example, growth-promoting substances, such as trace elements, for example iron, zinc, manganese and the like.

The medium is preferably so chosen as to exert a selection pressure and prevent the growth of cells which have not been transformed or have lost the hybrid vector. Thus, for example, an antibiotic is added to the medium if the hybrid vector contains an antibiotic resistance gene as marker. If, for instance, a host cell is used which is auxotrophic in an essential amino acid whereas the hybrid vector contains a gene coding for an enzyme which complements the host defect, a minimal medium deficient of said amino acid is used to culture the transformed cells.

Cells of higher eucaryotic origin such as mammalian cells are grown under tissue culture conditions using commercially available media, for example Dulbecco's modified Eagle medium (DMEM), minimum essential medium, RPMI 1640 medium and the like as mentioned above, optionally supplemented with growth-promoting substances and/or mammalian sera. Techniques for cell cultivation under tissue culture condition are well known in the art and include homogeneous suspension culture, e.g. in an airlift reactor or in a continuous stirrer reactor, or immobilized or entrapped cell culture, e.g. in hollow fibres, microcapsules, on agarose microbeads, porous glass beads, ceramic cartridges, or other microcarriers.

Culturing is effected by processes which are known in the art. The culture conditions, such as temperature, pH value of the medium and fermentation time, are chosen so that a maximum titer of the polypeptide or derivative of the invention is obtained. Thus, an *E. coli* or yeast strain is preferably cultured under aerobic conditions by submerged culture with shaking or stirring at a temperature of about 20° C. to 40° C., preferably at about 30° C., and a pH value of 4 to 8, preferably of about pH 7, for about 4 to 30 hours, preferably until maximum yields of the polypeptide or derivative of the invention are reached.

When the cell density has reached a sufficient value, the culture is interrupted and the polypeptide or derivative can be isolated. If the hybrid vector contains a suitable secretion signal sequence, the polypeptide or derivative is secreted by the transformed cell directly into the culture medium. Otherwise, the cells have to be destroyed, for example by treatment with a detergent such as SDS, NP-40™, Triton™ or deoxycholic acid, lysed with lysozyme or a similarly acting enzyme, or disrupted by an osmotic shock or ultrasound. Break-up of the cells will also be required if the signal sequence directs the secretion of the desired protein into the cell periplasm. If yeast is used as a host microorganism, the cell wall may be removed by enzymatic digestion with a glucosidase. Alternatively or additionally, mechanical forces, such as shearing forces (e.g. French press, Dyno mill and the like) or shaking with glass beads or aluminium oxide, or alternating freezing, for example in liquid nitrogen, and thawing, for example at 30° C. to 40° C., as well as ultra-sound can be used to break the cells.

The cell supernatant or the solution obtained after centrifugation of the mixture obtained after breaking the cells, which contains proteins, nucleic acids and other cell constituents, is enriched in proteins, including the polypeptides of the invention, in a manner which is known per se. Thus, for example, most of the non-protein constituents are removed by polyethyleneimine treatment and the proteins including the polypeptides and derivatives of the invention are precipitated, for example, by saturation of the solution with ammonium sulfate or with other salts. Otherwise, the cell supernatant or lysate is directly pre-purified by filtering through suitable membranes and/or with chromatographic methods, for example affinity chromatography.

The recombinant antibodies and the monoclonal antibodies according to the invention can be used for the qualitative and quantitative determination of the extracellular domain of the growth factor receptor c-erbB-2. This is especially useful for the monitoring of tumor progression, for the decision whether a tumor is amenable to treatment with the recombinant or monoclonal antibodies of the invention, and for monitoring the treatment of tumor with chemotherapy. Tumors considered are those over-expressing c-erbB-2, for example breast and ovarian tumors.

In general, the monoclonal and the recombinant antibodies according to the invention can be used in any of the known immunoassays which rely on the binding interaction between the antibodies and the antigen, i.e. the extracellular domain of the c-erbB-2 protein. Examples of such assays are radio-, enzyme, fluorescence, chemiluminescence, immunoprecipitation, latex agglutination, and hemagglutination immunoassays, and, in particular, immunostaining methods.

The antibodies according to the invention can be used as such or in the form of enzyme-conjugated derivatives in an enzyme immunoassay. Any of the known modifications of an enzyme immunoassay can be used, for example soluble phase (homogeneous) enzyme immunoassay, solid phase (heterogeneous) enzyme immunoassay, single enzyme immunoassay or double (sandwich) enzyme immunoassay with direct or indirect (competitive) determination of the c-erbB-2 protein.

An example of such an enzyme immunoassay is a sandwich enzyme immunoassay in which a suitable carrier, for example the plastic surface of a microtiter plate or of a test tube, e.g. of polystyrene, polypropylene or polyvinylchloride, glass or plastic beads, filter paper, dextran etc. cellulose acetate or nitrocellulose sheets, magnetic particles or the like, is coated with a monoclonal antibody of the invention by simple adsorption or optionally after activation of the carrier, for example with glutaraldehyde or cyanogen bromide. Then test solutions containing the soluble c-erbB-2 protein and finally single-chain recombinant antibodies of the invention comprising a detectable enzyme, e.g. alkaline phosphatase, are added. The amount of the soluble c-erbB-2 protein in the test solution is directly proportional to the amount of bound recombinant antibody and is determined by adding an enzyme substrate solution. The enzyme substrate reaction results, for example, in a color change which can be observed by eye or with optical measuring devices.

The antibodies according to the invention can be used as such or in the form of radioactively labelled derivatives in a radioimmunoassay (RIA). As described above for enzyme immunoassays, any of the known modifications of a radioimmunoassay can be used.

The tests are carried out in an analogous manner to the enzyme immunoassays described above using a radioactive label, e.g. ^{125}I, instead of an enzyme label. The amount of immune complex formed which corresponds to the amount of c-erbB-2 protein present in the test solutions is determined by measuring the radioactivity of the immune complex.

For immunostaining cryosections of cryopreserved biopsy material or paraffin embedded tissue sections are treated with a solution containing a recombinant antibody of the invention comprising a detectable enzyme. Bound recombinant antibody is detected by treatment with a suitable enzyme substrate, preferably an enzyme substrate which leads to a solid deposit (stain) at the site of the recombinant antibody of the invention. In place of recombinant antibodies comprising an enzyme, a recombinant antibody comprising streptavidin and a solution of a biotin-enzyme-conjugate may be used, which leads to higher enzyme concentration at the site of the antibody and hence increased sensitivity of the immunostaining method. The solid deposit of the enzyme substrate is detected by inspection with a microscope, for example with a fluorescence microscope, or by scanning the optical density at the wavelength of the stain.

The use according to the invention of recombinant and/or monoclonal antibodies as described hereinbefore for the determination of c-erbB-2 protein also includes other immunoassays known per se, for example immunofluorescence assays, latex agglutination with antibody-coated or antigen coated latex particles, hemagglutination with antibody-coated or antigen-coated red blood corpuscles, evanescent light assays using an antibody-coated optical fibre and other direct-acting immunosensors which convert the binding event into an electrical or optical signal, or the like.

The invention also concerns test kits for the qualitative and quantitative determination of c-erbB-2 protein comprising recombinant antibodies of the invention and/or monoclonal antibodies of the invention and, optionally, adjuncts.

Test kits according to the invention for an enzyme immunoassay contain, for example, a suitable carrier, optionally freeze-dried solutions of a monoclonal antibody, optionally freeze-dried or concentrated solutions of a recombinant antibody comprising an enzyme or streptavidin, solutions of an enzyme-biotin conjugate if a recombinant antibody comprising streptavidin is used, enzyme substrate in solid or dissolved form, standard solutions of c-erbB-2 protein, buffer solutions, and, optionally, polypeptides or detergents for preventing non-specific adsorption and aggregate formation, pipettes, reaction vessels, calibration curves, instruction manuals and the like.

Test kits according to the invention for immunostaining contain, for example, optionally freeze-dried or concentrated solutions of a recombinant antibody comprising an enzyme or streptavidin, solutions of an enzyme-biotin conjugate if a recombinant antibody comprising streptavidin is used, enzyme substrate in solid or dissolved form, buffer solutions, and, optionally, pipettes, reaction vessels, calibration curves, instruction manuals and the like.

The recombinant and monoclonal antibodies of the invention can be used for the qualitative and quantitative determination of c-erbB-2 protein. Due to the fact that the growth factor receptor c-erbB-2 is overexpressed in certain tumor types, for example breast and ovarian tumors, the antibodies are particularly well suited for detection and monitoring of the mentioned tumors. In addition, radiolabelled derivatives of the antibodies of the invention may be used for the in vivo localization of tumors in a patient using radioscanning techniques. To that end, radiolabelled derivatives of antibodies of the invention are injected into the patient, and the patient scanned with a gamma imager at regular intervals. Cells over-expressing the growth factor receptor c-erbB-2 will take up more radioactive antibodies than other tissue and will be clearly recognized by the gamma imaging camera. Preferentially recombinant or monoclonal antibodies labelled with ^{131}I or with ^{99m}Tc are used for radioscanning in amounts of 3 to 8 μg representing 15 to 30 μCi per kg body weight.

The antibodies of the invention can further be used for the isolation and purification of the c-erbB-2 protein from natural sources or from transformed host cells by immunoaffinity chromatography.

Furthermore, the monoclonal antibodies and the recombinant antibodies of the invention, in particular recombinant antibodies comprising an effector molecule, especially a toxin, in particular Pseudomonas exotoxin, are useful for the treatment of patients with tumors over-expressing the growth factor receptor c-erbB-2, for example breast or ovarian tumors. If it is desired, tumor therapy may comprise applying more than one, e.g. two different, antibodies of the invention, for example applying both FRP5 and FWP51. The recombinant antibodies comprising a phosphatase may be used in connection with a phosphorylated prodrug such as mitomycin phosphate or etoposide phosphate, thus enabling the conversion of the active drug to the prodrug at the site of the tumor.

The invention therefore also concerns pharmaceutical compositions for treating tumors over-expressing the growth factor receptor c-erbB-2 comprising a therapeutically effective amount of a recombinant antibody or of a monoclonal antibody according to the invention and a pharmaceutically acceptable carrier. Preferred are pharmaceutical compositions for parenteral application. Compositions for intramuscular, subcutaneous or intravenous application are e.g. isotonic aqueous solutions or suspensions, optionally prepared shortly before use from lyophilized or concentrated preparations. Suspensions in oil contain as oily component the vegetable, synthetic or semi-synthetic oils customary for injection purposes. The pharmaceutical compositions may be sterilized and contain adjuncts, e.g. for conserving, stabilizing, wetting, emulsifying or solubilizing the ingredients, salts for the regulation of the osmotic pressure, buffer

and/or compounds regulating the viscosity, e.g. sodium carboxycellulose, carboxymethylcellulose, sodium carboxymethylcellulose, dextran, polyvinylpyrrolidine or gelatine.

The pharmaceutical compositions of the invention contain from approximately 0.01% to approximately 50% of active ingredients. They may be in dosage unit form, such as ready-to-use ampoules or vials, or also in lyophylized solid form.

In general, the therapeutically effective dose for mammals is between approximately 5 and 25 μg of a recombinant antibody of the invention or of a monoclonal antibody of the invention per kg body weight depending on the type of antibody, the status of the patient and the mode of application. The specific mode of administration and the appropriate dosage will be selected by the attending physician taking into account the particulars of the patient, the state of the disease, the type of tumor treated, and the like. The pharmaceutical compositions of the invention are prepared by methods known in the art, e.g. by conventional mixing, dissolving, confectioning or lyophilizing processes. Pharmaceutical compositions for injection are processed, filled into ampoules or vials, and sealed under aseptic conditions according to methods known in the art.

The invention particularly concerns the monoclonal antibodies, the hybridoma cell lines, the recombinant single-chain antibodies, the recombinant DNAs, the transformed host cells, and the methods for the preparation thereof as described in the Examples. The following examples illustrate the invention but do not limit it to any extent.

Abbreviations

ATP	adenosine triphosphate
BSS	Earle's balanced salt solution
BSA	bovine serum albumin
DEAE	diethylaminoethyl
DMEM	Dulbecco's modified Eagle's medium
dNTP	deoxynucleotide triphosphate
DTT	dithiothreitol
EDTA	disodium ethylenediaminetetraacetate
EGF	epidermal growth factor
EGTA	ethyleneglycol-bis-(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid
FCS	fetal calf serum
HAT medium	hypoxanthine, aminopterin and thymidine medium
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
HT medium	hypoxanthine and thymidine medium
Ig	immunoglobulin
IPTG	isopropyl-β-thiogalactoside
MAb	monoclonal antibody
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PMSF	phenylmethylsulfonyl fluoride
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
Tris	Tris-(hydroxymethyl)-aminomethane
U	unit
V_L	light chain variable domain
V_H	heavy chain variable domain
XP	5-bromo-4-chloro-3-indolyl phosphate p-toluidine salt

EXAMPLES

Example 1

Preparation of Hybridoma Cell Lines FRP5, FSP16, FWP51 and FSP77

1.1 Source of antigen and immunization of Balb/c mice: The SKBR3 human breast tumor cell line (ATCC HTB 30), isolated in 1970 from a pleural effusion of a breast cancer patient, expresses approximately 1×10^6 molecules of the c-erbB-2 receptor protein per cell. 20×10^6 SKBR3 cells in PBS are injected subcutaneously and/or intraperitoneally into Balb/c mice. The cells are mixed 1:1 (v/v) with complete Freund's adjuvant. The injections are repeated a total of five times over the period of approximately 3 months replacing Freund's incomplete adjuvant for complete adjuvant. The final injection of cells is given three days before the fusion.

1.2 Cell fusion: Immunized mice are sacrificed and their splenocytes fused according to conventional methods (Koehler & Milstein, Nature 256: 495, 1976). Spleen cells are mixed at a 5:1 to 10:1 ratio with the fusion partner, the mouse myeloma cell line PAI (Stoker et al., Research Disclosure #21713, 1982), in the presence of 41% polyethylene glycol 4000 (Merck). Fused cells are plated at a density of 1×10^6 cells per well in 24-well microtiter plates on peritoneal macrophages and fed 3 times per week with standard HAT selection medium for 2 weeks followed by 2 weeks of HT medium. When the growth of hybridoma cells becomes visible, the supernatants are screened as described in Example 1.3. Positive hybridomas are cloned and stored.

1.3 Antibody detection in hybridoma supernatants: Culture fluids of growing hybridomas are tested for the presence of anti-c-erbB-2 antibody using a protocol involving two steps, immunofluorescence and immunoprecipitation.

1.3.1 Immunofluorescence: In the first step, hybridoma supernatants are tested for their immunofluorescent staining of mouse cells expressing high levels of the human c-erbB-2 protein. To isolate these cells the HC11 mouse mammary epithelial cell line (Ball at al., EMBO J. 7: 2089, 1988) is transfected according to conventional, previously described methods (Graham & van der Eb, Virology 52: 456, 1973) with a plasmid expressing the human c-erbB-2 protein (Masuko et al., Jpn. Cancer Res. 80: 10, 1989) and with the plasmid pSV2neo (Southern & Berg, J. Mol. Appl. Genet. 1: 327, 1982) which encodes the gene for resistance to the drug G418. Transfected cells are selected 2 weeks in medium containing 200 μg/ml G418 (Geneticin, Gibco-BRL). Individual clones are selected and analyzed for expression of the human c-erbB-2 protein using conventional protein blotting techniques (Towbin et al., Proc. Natl. Acad. Sci. USA 76: 4350, 1979). A clone expressing high levels of the human c-erbB-2 protein (clone R1#11) is selected and used in the immunofluorescent assay. Non-transfected HC11 cells serve as control cells.

The assay is done in the following manner: The cells (R1#11 or HC11) are grown in RPMI medium containing 8% heat inactivated FCS (Amimed), 10 ng/ml EGF (Inotech) and 5 μg/ml insulin (Sigma) for 1–2 days on fibronectin (Boehringer Mannheim) coated cover slips. Fibronectin coated cover slips are prepared and stored at room temperature and they are used routinely for screening. The coverslips are rinsed in PBS containing calcium and magnesium and fixed by treatment for 10 min with 3.7% formaldehyde (v/v in PBS). To reduce the non-specific binding the coverslips are incubated 20 min in PBS containing 3% BSA (Sigma). The coverslips are washed in PBS and in water, then allowed to dry at room temperature. 20–30 μl of hybridoma supernatants are added to circled areas on a coverslip which is incubated 1–2 h at room temperature in a humified atmosphere. The coverslips are then washed three times with PBS containing 0.05% Triton-X 100™ (Fluka) and incubated an additional hour with anti-mouse Ig, fluorescein-linked whole antibody from sheep (Amersham). After three washes with PBS and one wash with water the

cells are screened for fluorescence using a fluorescence microscope and a water immersion lens. Those hybridoma supernatants which are positive are screened in the second step described in Example 1.3.2.

1.3.2 Immunoprecipitation and protein blotting analysis: The SKBR3 human breast tumor cells express approximately 1×10^6 molecules of the c-erbB-2 protein per cell. A cell lysate is prepared by extracting approximately 4×10^6 cells in 1 ml of buffer containing 1% Triton-X100™ (Fluka), 50 mM Tris-HCl, pH 7.5, 5 mM EGTA, 0.15M NaCl, 1 mM PMSF (Boehringer Mannheim), 80 μg/ml aprotinin (Boehringer Mannheim), 50 μg/ml leupeptin (Boehringer Mannheim), and 4 μg/ml pepstalin (Boehringer Mannheim). 200–500 μl supernatant of hybridomas which are positive in the immunofluorescence assay described in Example 1.3.1 are incubated with 100 μl of the SKBR3 extract (2.5–4.0 mg/ml). This amount of extract contains approximately 50–100 ng of c-erbB-2 protein. The hybridoma supernatants and SKBR3 extract are incubated overnight on ice, then 1 μl of the IgG fraction of sheep anti-mouse Ig (ICN Immunobiologicals) is added. The complexes are collected by the addition of Protein-A Sepharose™ (Pharmacia), washed with TNET (140 mM NaCl, 50 mM Tris-HCl, pH 7.5, 5 mM EDTA, 1% Triton X-100™) and water, boiled in sample buffer (80 mM Tris-HCl, pH 6.8, 0.2% SDS, 10% glycerol) and the supernatants loaded onto 8% SDS-PAGE. The proteins are electrophoresed and blotted onto PVDF membranes (Millipore) using a technique originally described by Towbin et al. (Proc. Natl. Acad. Sci. USA 76: 4350, 1979) with some modifications. The proteins are transferred using a semi-dry blotter (G. Frobel, Model 1004.01) following the instructions of the manufacturer. The membranes are blocked in PBS containing 0.5% gelatin (Merck) for 1 h at 37° C. The membranes are washed twice for 5 min at 37° C. in PTG (PBS containing 0.02% gelatin (Merck) and 0.25% Triton-X100™ (Fluka)). The c-erbB-2 protein is detected by incubating the membrane 45 min at 37° C. in PTG containing an antiserum which is raised against the carboxy terminal 13 amino acids of the c-erbB-2 protein (Gullick et al., Int. J. Cancer 40: 246, 1987, antiserum 21N). The membranes are washed 3 times for 5 min at 37° C. in PTG. The membrane-bound 21N antiserum is detected by incubating the membrane in PTG containing 0.1 μC/ml ^{125}I-labeled protein-A (Amersham). The membranes are washed 4 times for 5 min at 37° C. in PTG and exposed to X-ray film. The hybridomas whose supernatants are able to specifically immunoprecipitate the c-erbB-2 protein are grown for single cell cloning and further characterization described below.

Example 2

Characterization of c-erbB-2 Specific MAbs

2.1 Hybridoma storage and processing: Hybridoma FRP5, FSP16, FWP51 and FSP77 secreting anti-c-erbB-2 MAb FRP5, FSP16, FWP51 and FSP77, respectively, can be grown in culture, frozen at –80° C. or in liquid nitrogen and recultivated. The cells are cloned by the method of limiting dilution and have been deposited with the European Collection of Animal Cell Lines in England. The hybridoma cell lines have the following access numbers: FRP5: 90112115, FSP16: 90112116, FSP77: 90112117, FWP51: 90112118. The cells are expanded by forming ascites in Balb/c mice primed with pristane. The antibodies are purified from the ascites by ammonium sulfate precipitation and ion exchange chromatography on DE 52 DEAE-cellulose columns (Whatman). Purified MAbs are stored in PBS at –80° C.

2.2 Isotyping of the MAbs: The isotype of the MAbs FRP5, FSP16, FWP51 and FSP77 is determined by ELISA analysis with rabbit antisera to mouse Ig classes and subclasses (Biorad Mouse Typer TMSub Isotyping Kit™) as per manufacturer's suggested procedure. MAbs FRP5, FWP51, and FSP77 are of the IgG1 isotype, while FSP16 is of the IgG2b isotype. The light chains of all the MAbs are of the kappa type.

2.3 Flow cytometry: A FACS analysis using the c-erbB-2 specific MAbs is carried out as follows: SKBR3 human breast tumor cells are trypsinized, washed in FACS medium (BSS containing 10 μM sodium azide, 4% FCS and 25 mM EDTA), and 1×10^6 cells are resuspended in 100 μl of FACS medium. Non-specific binding sites are blocked by incubating the cells 10 min at room temperature with 5 μl of goat serum. The SKBR3 cells are collected by centrifugation, resuspended in 50 μl of a 1:2 dilution of the supernatant made in FACS medium and incubated 45 min on ice. The cells are washed with 4 ml FACS medium, collected by centrifugation, resuspended in 50 μl of FACS medium containing a 1:20 dilution of anti-mouse Ig, fluorescein-linked whole antibody from sheep (Amersham), and incubated for 30 min on ice. 4 ml of FACS medium are added, the cells are collected by centrifugation, resuspended in 100 μl of FACS medium and analyzed without fixation for their fluorescence in a Becton-Dickinson FACScan™. As a control, SKBR3 cells are incubated with a non-reacting IgG1 MAb (1236S31-3). The FACS analysis shows that the SKBR3 cells treated with MAb FRP5, FSP16, FWP51, and FSP77 have a higher fluorescence than cells treated with the control MAb. These results show that the MAbs bind to the extracellular domain of the c-erbB-2 protein.

2.4 Binding domain of c-erbB-2 specific MAbs: MAbs FRP5 and FSP77 are covalently linked with ^{125}I (as carrier free sodium ^{125}iodide, Amersham) to a specific activity of 1 μC/μg using Iodogen (1,3,4,6-tetrachloro-3a,6a-diphenylglycouril, Sigma) according to a standard protocol (Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory, 1988, p. 330). Competition experiments are conducted by incubating SKBR3 cells ($0.5–1\times10^5$ cells per 15 mm well, Nunclon™ 4-well multidish) with 250 μl RIA buffer (120 mM NaCl, 50 mM HEPES, pH 7.8, 1 mM EDTA, 2% BSA) containing labeled FRP5 or FSP77 and varying amounts of unlabeled MAb FRP5, FSP16, FWP51 and FSP77 for 2 h at 4° C. The cells are washed 5 times with the RIA buffer, solubilized in 0.5 ml 1% Triton X-100™, 10% glycerol, 20 mM HEPES, pH 7.4, for 30 min at room temperature and the bound radioactivity is measured in a gamma counter. The results show that MAbs FRP5 and FSP16 compete with each other for binding to SKBR3 cells which suggests that these 2 MAbs bind to the same domain on the c-erbB-2 protein. MAbs FWP51 and FSP77 neither compete with each other nor with FRP5 or FSP16 for binding to the c-erbB-2 protein. In conclusion, the panel of 4 MAbs bind to 3 different domains of the extracellular portion of the c-erbB-2 membrane receptor tyrosine kinase.

Example 3

Isolation of RNA from the Hybridoma Cell Line FRP5

3.1 Growth of FRP5 cells: FRP5 hybridoma cells (1×10^8) are grown in suspension culture at 37° C. in DMEM (Seromed) further containing 10% FCS (Amimed), 1 mM sodium pyruvate (Seromed), 2 mM glutamine (Seromed), 50 μM 2-mercaptoethanol and 100 μg/ml of gentamycin

(Seromed) in a humidified atmosphere of air and 7.5% CO_2 in 175 cm tissue culture flasks (Falcon 3028). The cells are harvested by centrifugation, washed once in PBS, flash frozen in liquid nitrogen and kept frozen as a pellet at −80° C. in a clean, sterile plastic capped tube.

3.2 Extraction of total cellular RNA from FRP5 cells: Total RNA is extracted using the acid guanidinium thiocyanate-phenol-chloroform method described by Chomczynski & Sacchi (Anal. Biochem. 162: 156, 1987). Cell pellets of FRP5 cells (1×10^8) are thawed directly in the tube in the presence of 10 ml of denaturing solution (4M guanidinium thiocyanate (Fluka), 25 mM sodium citrate, pH 7.0, 0.5% N-lauroylsarcosine (Sigma), 0.1M 2-mercaptoethanol). The solution is homogenized at room temperature. Sequentially, 1 ml of 2M sodium acetate, pH 4, 10 ml of phenol (water saturated) and 2 ml of chloroform-isoamyl alcohol mixture (49:1) are added to the homogenate. The final suspension is shaken vigorously for 10 sec and cooled on ice for 15 min. The samples are centrifuged at 10,000×g for 20 min at 4° C. After centrifugation, RNA which is present in the aqueous phase is mixed with 10 ml of isopropanol and placed at −20° C. for 1 h. The RNA precipitate is collected by centrifugation, the pellet dissolved in 3 ml water and the RNA reprecipitated by addition of 1 volume of isopropanol at −20° C. After centrifugation and washing the pellet in ethanol, the final pellet of RNA is dissolved in water. The method yields approximately 300 µg of total cellular RNA. The final purified material is stored frozen at −20° C.

3.3 Isolation of poly(A) containing RNA: Poly(A) containing RNA is selected from total RNA by chromatography on oligo(dT)-cellulose (Boehringer Mannheim) as described originally by Edmonds et al. (Proc. Natl. Acad. Sci. USA 68: 1336, 1971) and modified by Maniatis et al. (Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, 1982, p. 197). The poly(A)-containing RNA is prepared as described in the published procedure with the exception that the RNA is eluted from the oligo(dT)cellulose with water rather than SDS-containing buffer. The poly(A)-containing RNA is precipitated with ethanol and collected by centrifugation. The yield of poly(A)-containing RNA is approximately 30 µg from 300 µg of total cellular RNA. The final purified material is stored frozen at −20° C.

Example 4

Cloning of Functional Heavy and Light Chain Rearrangements from the FRP5 Hybridoma Cell Line

Poly(A)-containing RNA isolated from FRP5 hybridoma cells as described in Example 3.3 provides the source for cDNA synthesis and subsequent amplification of V-region minigenes. Amplification products of the expected size are purified from agarose gels and cloned into appropriate vectors. Functional rearrangements are identified by sequencing.

4.1 Oligonucleotides: MCK2 is designed to be complementary to a region in the murine immunoglobulin κ (kappa) constant minigene.

5'-TCACTGGATGGTGGGAAGATGGA-3'

MCHC2 is designed to be complementary to a region in the murine immunoglobulin γ1 constant minigene.

5'-AGATCCAGGGGCCAGTGGATAGA-3'

The oligonucleotides VH1FOR, VH1BACK, VK1FOR, and VK1BACK are designed by Orlandi et al. (Proc. Natl. Acad. Sci. USA 86: 3833, 1989) to match consensus sequences.

VH1FOR: 5'-TGAGGAGACGGTGACCGTGGTCCCTTG-GCCCCAG-3'

VH1BACK: 5'-AGGT(C/G)(C/A)A(G/A)CTGCAG(G/C)AGTC(T/A)GG-3'

VK1FOR: 5'-GTTAGATCTCCAGCTTGGT(C/G)C(C/G)-3'

VK1BACK: 5'-GACATTCAGCTGACCCAGTCTCCA-3'

4.2 cDNA synthesis: 55 ng of poly(A)-containing RNA is dissolved in a buffer containing 50 mM Tris-HCl, pH 8.3, mM magnesium chloride, 10 mM DTT, 75 mM KCl, 400 µM dNTPs (N=G, A, T and C), 100 µg BSA (molecular biology grade, Boehringer Mannheim), 100 U RNAse inhibitor (Boehringer Mannheim), 25 pmol MCK2 and 25 pmol MCHC2. The RNA is denatured at 70° C. for 5 min and then chilled on ice for 2 min. After addition of 200 U of MMLV reverse transcriptase (Gibco, BRL) cDNA synthesis is achieved by incubation for 1 h at 37° C.

4.3 Polymerase chain reaction: One tenth of the cDNA reaction is used for DNA amplification in buffer containing 10 mM Tris-HCl, pH 8.3, 1.5 mM $MgCl_2$, 50 mM KCl, 10 mM β-mercaptoethanol, 200 µM dNTPs (N=G, A, T and C), 0.05% Tween-20™ (Merck), 0.05% NP-40™ (Merck), 10% DMSO (Merck), 25 pmol oligonucleotide 1 (see below), 25 pmol oligonucleotide 2 (see below) and 2.5 U Amplitaq™ DNA polymerase (Perkin Elmer Cetus). Taq polymerase is added after initial denaturation at 93° C. for 1 min and subsequent annealing at 37° C. In the first 4 cycles primer extension is performed at 71° C. for 0.2 min, denaturation at 93° C. for 0.01 min and annealing at 37° C. for 0.2 min. For the last 25 cycles the annealing temperature is raised to 62° C. Finally, amplification is completed by a 3 min primer extension step at 71° C.

PCR Product	oligonucleotide 1	oligonucleotide 2
HC	MCHC2	VH1BACK
H	VH1FOR	VH1BACK
LC	MCK2	VK1BACK
L	VK1FOR	VK1BACK

4.4 Modification and purification: Amplified material is extracted with $CHCl_3$ and precipitated with ethanol in the presence of 200 mM LiCl. To facilitate cloning, blunt ends are created by a 3 min treatment with 1 U T4 DNA polymerase (Boehringer Mannheim) in 66 mM Tris-acetate, pH 7.9, 132 mM potassium acetate, 20 mM magnesium acetate, 1 mM DTT, 200 µg/ml BSA (molecular biology grade, Boehringer Mannheim), and 400 µM dNTPs (N=G, A, T and C). The polymerase is inactivated by heating for 15 min at 65° C. before phosphorylation of the DNA with 10 U T4 polynucleotide kinase (Pharmacia) at 37° C. for 1 h. For this purpose the buffer is adjusted to 50 mM EDTA and 1 mM ATP. The modified amplification products are separated on a 1.2% (w/v) agarose gel (ultra pure DNA grade agarose, Biorad) and DNA of the expected size is eluted by means of DEAE NA 45 membranes (Schleicher & Schuell).

4.5 Ligation: Bluescript™ KS+ (70 ng) linearized with XbaI, treated with Klenow DNA polymerase (Boehringer Mannheim) to give blunt ends and dephosphorylated with calf intestinal phosphatase, and 30 ng of purified amplification product are ligated using 0.5 U T4 DNA ligase (New

England Biolabs) in 50 mM Tris-HCl, pH 7.8, 10 mM magnesium chloride, 10 mM DTT, and 0.8 mM ATP overnight at 16° C. One half of the ligation mixture is used to transform *E. coli* K803 to obtain ampicillin resistant colonies. These are screened for the desired ligation products using a NaOH based plasmid "miniprep" method (Maniatis et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, 1982). The following plasmids are obtained:

PCR product	Plasmid clones
HC	pMZ15/1
	pMZ15/2
H	pMZ16/1
	pMZ16/2
L	pMZ17/1
	pMZ17/2
LC	pMZ18/1
	pMZ18/2

4.6 Sequencing: Sequencing is done using Sequenase™ kits (United States Biochemicals) with T3 and T7 oligonucleotide primers according to procedures provided by the manufacturer.

Plasmid pMZ17/1 contains a non-functional rearrangement. Plasmid pMZ17/2 contains an Ig-unrelated sequence. Plasmids pMZ18/1 (SEQ ID NO:2) and pMZ18/2 contain identical functional FRP5 kappa light chain variable domain inserts. Plasmids pMZ16/1 (SEQ ID NO:1) and pMZ16/2 contain identical functional FRP5 heavy chain variable domain inserts. Plasmids pMZ15/1 and pMZ15/2 also contain FRP5 heavy chain variable domain inserts together with some constant region DNA. Plasmids pMZ16/1 and pMZ18/1 are used as a source for further subcloning steps.

Example 5

Construction of the MAb FRP5 Single-chain Fv Gene

5.1 Construction and sequence of a cloning linker for the heavy and light chain variable domain cDNAs: Using oligonucleotides, a linker sequence which allows the cloning of PCR amplified mouse heavy chain variable domain cDNA as a PstI/BstEII fragment and of PCR amplified mouse kappa light chain variable domain cDNA as a PvuI/BglII fragment is constructed. This creates an open reading frame in which heavy and light chain variable domains are connected by a sequence coding for the 15 amino acid stretch Gly-Gly-Gly-Gly-Ser-Gly-Gly-Gly-Gly-Ser-Gly-Gly-Gly-Gly-Ser. This amino acid linker has been shown to allow correct folding of an antigen binding domain present in heavy and light chain variable domains in a single-chain Fv (Huston et al., Proc. Natl. Acad. Sci. USA 85: 5879, 1988).

For the construction of the cloning linker the 6 complementary oligonucleotides 1A, 1B, 2A, 2B, 3A, 3B are used.

1A: 5'-CAAGCTYCTCAGGTACAACTGCAGGAG-GTCACCGTTFCCTCTGGCGG-3'

1B: 5'-GAAACGGTGACCTCCTGCAGTFGTACCT-GAGAAGCTTGCATG-3'

2A: 5'-TGGCGGTYCTGGTGGCGGTGGCTCCG-GCGGTGGCGGTTCTGAC-3'

2B: 5'-GCCACCGCCGGAGCCACCGCCACCA-GAACCGCCACCGCCAGAG-3'

3A: 5'-ATCCAGCTGGAGATCTAGCTGATCAAAGCT-3'

3B: 5'-CTAGAGCTTFGATCAGCTAGATCTC-CAGCTGGATGTCAGAACC-3'

40 pM of oligonucleotides 1B, 2A, 2B, 3A are phosphorylated at the 5' end using T4 polynucleotide kinase (Boehringer Mannheim) in four separate reactions in a total volume of 20 µl following the method described by Maniatis et al. (Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, 1982). Oligonucleotides 1A and 3B are not phosphorylated in order to avoid self ligation of the linker in the final ligation reaction. After the kinase reaction, the enzyme is inactivated by incubation at 70° C. for 30 min. In three separate reactions, each containing 40 pM of two oligonucleotides in a total volume of 40 µl, non-phosphorylated 1A and phosphorylated 1B, phosphorylated 2A and phosphorylated 2B, and phosphorylated 3A and non-phosphorylated 3B are mixed. Hybridization of the oligonucleotides in the three reactions is carried out by heating to 95° C. for 5 min, incubation at 65° C. for 5 min and slowly cooling to room temperature. 10 µl from each of the three reactions are mixed, 4 µl of 10×ligation buffer (Boehringer) and 4 units of T4 DNA ligase (Boehringer) are added and the total volume is adjusted to 40 µl with sterile water. The annealed pairs of oligonucleotides are ligated into one linker sequence for 16 h at 14° C. The reaction mixture is extracted with an equal volume of phenol/chloroform (1:1) followed by re-extraction of the aqueous phase with an equal volume of chloroform/isoamylalcohol (24:1). The aqueous phase is collected, 0.1 volumes of 3M sodium acetate pH 4.8 and 2 volumes of ethanol are added, and the DNA is precipitated at −70° C. for 4 h and collected by centrifugation. The resulting linker sequence has a SphI and a XbaI adaptor end. It is ligated to SphI and XbaI digested pUC19 in a reaction containing 100 ng of ligated linker and 200 ng of SphI/XbaI digested pUC19. After transformation into *E. coli* XL1 Blue™ (Stratagene), plasmid DNA from 4 independent colonies is isolated by the alkaline lysis minipreparations method (Maniatis et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, 1982). The DNA sequence of the linker cloned in pUC19 is determined by sequencing double stranded DNA in both directions with Sequenase II (United States Biochemicals) and pUC universal and reverse primers (Boehringer) following the manufacturer's protocol. Three out of the four recombinant pUC19 isolates sequenced contain the correct linker sequence. One of them is designated pWW19 and used in the further experiments. The sequence is shown in SEQ ID NO:3.

5.2 Preparation of a plasmid for the subcloning of variable domains: The Fv cloning linker sequence is derived as a 144 bp HindIII/SacI fragment from pWW19 and inserted into HindIII/SacI digested Bluescript™ KS+ (ex PvuII) (Stratagene) which contains no PvuII restriction sites. The resulting plasmid, pWW15, allows cloning of heavy and light chain variable domains as PstI/BstEII and PvuII/BglII fragments, respectively.

5.2.1 Subcloning of the FRP5 heavy chain variable domain: Plasmid pMZ16/1 is digested with PstI and BstEII and the 338 bp heavy chain variable domain fragment of FRP5 is isolated. It is cloned into PstI/BstEII digested pWW19 yielding the plasmid pWW31.

5.2.2 Mutation of the FRP5 light chain variable domain and assembly of the Fv fusion gene: To facilitate subcloning of the FRP5 light chain variable domain into the Fv cloning linker, a PvuII restriction site and a BglII restriction site are introduced at the 5' and 3' ends, respectively, of the coding

region. The FRP5 light chain variable domain coding region is isolated as a SacI/BamHI fragment from pMZ18/1. SacI and BamHI are restriction sites of the Bluescript™ polylinker present in pMZ18/I. The fragment contains the complete light chain variable domain fragment of 392 bp amplified by PCR using the oligonucleotide MCK2 (see above). This fragment is mutated and amplified by PCR using the oligonucleotides

V_L5': 5'-GACATTCAGCTGACCCAG-3' and

V_L3': 5'-GCCCGTYAGATCTCCAATTTTGTCCCCGAG-3'

for the introduction of a PvuII restriction site at the 5' end (V_L5') and a BglII restriction site at the 3' end (V_L3') of the kappa light chain variable domain DNA. 20 ng of the FRP5 variable light chain SacI/BamHI fragment are used as a template in a 100 µl reaction following the PCR conditions described in Example 4.3. The amplified and mutated fragment is isolated after PvuII/BglII digestion as a 309 bp fragment from a 1.5% agarose gel and cloned into PvuII/BglII digested pWW15 generating plasmid pWW41. The FRP5 kappa light chain variable domain is isolated as a BstEII/XbaI fragment from pWW41 and inserted into BstEII/XbaI digested pWW31. Thus the FRP5 heavy chain variable domain in pWW31 and the FRP5 kappa light chain variable domain are fused to one open reading frame. Double stranded DNA of three independent clones is sequenced with Sequenase II™ kit (United Biochemicals) in both orientations using pUC universal and reverse primers (Boehringer) following the manufacturer's protocol. One of the plasmids carrying the FRP5 heavy chain variable domain fused to the mutated FRP5 light chain variable domain is selected and designated pWW52. The sequence of the HindIII/XbaI insert in plasmid pWW52 is shown in SEQ ID NO:4 and 5.

Example 6

Construction of a Single-chain Fv-phosphatase Fusion Gene Expression Plasmid

The MAb FRP5 single-chain Fv gene is fused to the bacterial alkaline phosphatase. This chimeric gene encodes a bifunctional molecule which retains binding activity to the c-erbB-2 protein and has enzymatic activity.

6.1 Mutation of the single-chain Fv(FRP5) gene: To allow gene fusion between the single-chain Fv(FRP5) encoding gene from pWW52 and the alkaline phosphatase gene phoA the stop codon at sequence position 729 to 731 in pWW52 (see Example 5.2.3) is deleted as follows: Plasmid DNA of pWW52 is digested with BstEII and BglII and the linker sequence and FRP5 light chain variable domain encoding fragment is isolated. In another digestion, pWW52 is cleaved with BstEII and BclI. Thus, the large fragment containing vector sequences and the FRP5 heavy chain variable domain encoding sequence is isolated. The BstEII/BglII V_L fragment is now inserted into BstEII/BclI cleaved pWW52 containing V_H. In the resulting plasmid, pWW53, the BglII/BclI junction is determined by sequencing double stranded DNA as described above.

Sequence of the BglII/BclI junction in pWW53 (position numbers correspond to position numbers of the HindIII/XbaI insert in plasmid pWW52, SEQ ID NO:4 and 5):

BglII/BclI

ACA AAA TTG GAG ATC AAA GCT CTA GA

714–728 | 738–748

6.2 Mutation of the *E.coli* alkaline phosphatase gene phoA: For the construction of the Fv(FRP5)-phoA fusion gene the *E. coli* alkaline phosphatase gene phoA is mutated to generate a XbaI cleavage site in the coding region of phoA near the N terminus of the mature protein and a SacI cleavage site in the 3' untranslated region of phoA. This step facilitates the cloning of the mutated fragment. A pBR322 derivative carrying the recombinant transposon TnPhoA (Manoil & Beckwith, Proc. Natl. Acad. Sci. USA 82: 8129, 1985) is linearized by BglII cleavage. 20 ng of the linearized template DNA is used for a 100 µl PCR reaction carried out as described previously using oligonucleotides PhoA5' and PhoA3' as primers 1 and 2.

PhoA5': 5'-CCCTCTAGAGCCTGTTCTGGAAAAC-3'

PhoA3': 5'-CCCGAGCTCTGCCATTAAG-3'

Following XbaI/SacI digestion of the PCR products, a 1419 bp fragment is isolated from a 1.5% agarose gel and inserted into XbaI/SacI digested plasmid pUC19. Ligation is carried out as described above. Ligated DNA is transformed into *E. coli* XL1 Blue™ (Stratagene). Thus, the open reading frame of the mutated phoA gene is fused in frame to the lacZ open reading frame of pUC19. To show that the mutated phoA gene expresses functional alkaline phosphatase, recombinant clones are plated onto LB agar plates containing 100 µg/ml ampicillin, 0.5 mM IPTG (Sigma), and 40 µg/ml XP (Boehringer). Following induction of the lac promoter of pUC 19, a lacZ-phoA fusion protein is expressed. The phosphatase activity of this fusion protein converts the indicator XP to a blue dye. One of the blue colonies is isolated and the presence of the introduced restriction sites is confirmed by digestion of miniprep DNA with XbaI and SacI. Partial 5' and 3' DNA sequences of the mutated phoA gene are obtained by sequencing double stranded DNA as described above. The DNA sequences are included in the assembly of the final Fv(FRP5)-phoA fusion gene sequence shown in SEQ ID NO:6 and 7. The isolated plasmid is designated pWW61 and used for further subcloning steps.

6.3 Construction of a FRP5 Fv-phoA expression plasmid: From plasmid pWW19 (see Example 5.1.2) the cloning linker sequence is isolated as a HindIII/EcoRI fragment and inserted into HindIII/EcoRI digested plasmid pINIII-ompA-Hind (Rentier-Delrue et al., Nucl. Acids Res. 16: 8726, 1988) leading to plasmid pWW16.

From pWW61 (see Example 6.2) the mutated phoA gene is isolated as a XbaI/SacI fragment and inserted into XbaI/SacI digested pWW53. The resulting plasmid, pWW615, carries the Fv(FRP5) gene fused in frame to the mutated alkaline phosphatase gene. The Fv(FRP5)-phoA gene is isolated as a HindIII/SacI fragment from pWW615 and inserted into HindIII/SacI digested plasmid pWW16. This leads to the production of the Fv(FRP5)-phoA expression plasmid pWW616 (see below). All ligations are carried out as described above. Recombinant plasmids are transformed into *E. coli* XL1 Blue™ (Stratagene). The constructs are confirmed by restriction enzyme analysis of plasmid DNA isolated by an alkaline mini preparation method (Maniatis et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, 1982).

In this construct the Fv single-chain antibody of FRP5, genetically fused to the alkaline phosphatase phoA, can be expressed in *E. coli* following induction with IPTG. The recombinant protein carries the *E. coli* outer membrane protein A (ompA) signal sequence at the N terminus (encoded by the pINIII-ompA-Hind vector) to facilitate secretion of the protein into the periplasmic space of *E. coli* expressor cells.

The sequence of the Fv(FRP5)-phoA fusion gene in expression plasmid pWW616 is shown in SEQ ID NO:6 and 7. Part of the phoA sequence is assembled from Chang et al., Gene 44: 121, 1986.

Example 7

Expression of Fv(FRP5)-phoA in *E. coli*

Plasmid pWW616 is transformed into the phoA negative *E. coli* strain CC118 (Manoil & Beckwith, Proc. Natl. Acad. Sci. USA 82: 8129, 1985). A recombinant single colony is grown overnight in 50 ml LB medium containing 70 μg/ml ampicillin. The overnight culture is diluted 1:10 in 500 ml fresh LB medium containing 70 μg/ml ampicillin and grown at 37° C. to an OD_{550} of 0.1. IPTG is added to a final concentration of 2 mM and expression is induced for 1.5 h at 37° C. The cells are harvested at 4° C. by centrifugation at 4000 rpm for 25 min in a Beckman GPKR centrifuge. The supernantant of CC118/pWW616 is set aside on ice for preparation of Fv(FRP5)-phoA, see Example 7.2.

7.1 Isolation of Fv(FRP5)-phoA from the periplasmic proteins of CC118/pWW616: The bacterial pellet is suspended in 10 ml TES buffer (0.2M Tris-HCl, pH 8.0, 0.5 mM EDTA, 0.5M sucrose) and kept on ice for 10 min. After centrifugation at 4° C. for 10 min at 5000 rpm in a Heraeus minifuge, the supernatant is discarded and the washed pellet is suspended in 15 ml ice-cold TES, diluted (1:4) with water. The cells are kept on ice for 30 min and recentrifuged as above. The supernatant containing periplasmic proteins is recentrifuged at 45,000xg for 15 min in a Beckman TL100 ultracentrifuge. The periplasmic extract is concentrated in an Amersham ultrafiltration unit through a YM10 membrane to a final volume of 2 ml. Following fivefold dilutions with PBS and reconcentration through the YM10 membrane five times, the 1:4 diluted TES buffer of the periplasmic extract is exchanged with PBS. NaN_3 and protease inhibitors are added to the periplasmic proteins (2 ml in PBS) to the final concentration of 0.02% NaN_3, 0.1 mM PMSF, 2 μg/ml aprotinin, 1 μg/ml leupeptin, and 1 μg/ml pepstatin. The periplasmic extract is stored at 4° C.

7.2 Isolation of Fv(FRP5)-phoA from the concentrated supernatant of *E. coli* CC118/pWW616 cultures: The supernatant (500 ml) of the induced *E. coli* culture CC118/pWW616 is filtered through a 0.45 μm membrane. The filtrate is concentrated in an Amicon ultrafiltration unit through a YM10 membrane to a final volume of 10 ml in PBS as described above. NaN_3 and protease inhibitors are added to the concentrated supernatant to the final concentrations indicated above. The concentration of Fv(FRP5)-phoA in the extracts is determined by densitometry in comparison to BSA standards of coomassie stained 9% SDS-PAGE gels.

Example 8

Activity of Fv(FRP5)-phoA

8.1 Detection of c-erbB-2 in SKBR3 breast tumor cells by immunostaining using Fv(FRP5)-phoA: The Fv domain of Fv(FRP5)-phoA enables the molecule to bind to the extracellular domain of the c-erbB-2 protein. Bound Fv(FRP5)-phoA can be visualized by staining procedures using color substrates for the detection of alkaline phosphatase activity.

8.1.1 Fixation of cells: SKBR3 human breast tumor cells carrying about 1×10^6 c-erbB-2 receptors per cell are grown on fibronectin coated glass cover slips. The cells are washed twice with PBS and then fixed with PBS/3.7% formaldehyde at room temperature for 30 min. The fixed cells are washed three times with PBS at room temperature. Unspecific binding sites are blocked by incubating the cells for 1 h with PBS/3% BSA at 37° C. in a humid incubator. The cells are then washed twice with PBS.

8.1.2 Pretreatment of Fv(FRP5)-phoA: Alkaline phosphatase phoA from *E. coli* must be dimerized to be enzymatically active. In the periplasm of *E. coli* natural phoA is dimerized, i.e. two molecules of phoA are held together by two Zn^{2+} ions. The Fv(FRP5)-phoA is also produced as a dimer in *E. coli*. To increase binding of Fv(FRP5)-phoA to the antigen, the dimers are monomerized by adding EGTA to the solution. This step removes Zn^{2+} from the solution. Monomerized phosphatase can be re-dimerized by the addition of Zn^{2+}. EGTA is added to a final concentration of 5 mM to 200 μl of 40xconcentrated supernatant or periplasmic proteins from CC118/pWW616 (see above). The solution is incubated at 37° C. for 1 h just before use in the immunoassay.

8.1.3 Staining of cells: After blocking with PBS/3% BSA (see above) fixed cells are incubated for 1 h with pretreated Fv(FRP5)-phoA at a concentration of 1 μg/ml at 37° C. in a humidified incubator. The cells are washed three times with PBS at room temperature. The staining solution consists of 300 μl naphtol AS-MX™ phosphate (Sigma, 13 mg/ml in dimethyl formamide), 8 mg of levamisole (Sigma), and 10 mg of Fast Red TR™ salt (Sigma) added to 9.7 ml of 100 mM Tris-HCl, pH 8.2, 1 mM $ZnCl_2$. This mixture is prepared and filtered through a 0.45 μm filter immediately before use. $ZnCl_2$ is added to the staining solution to allow re-dimerization of bound Fv(FRP5)-phoA and thereby activating the alkaline phosphatase. Cells are incubated in the Fast Red™ staining solution for 15 min at room temperature. The phosphatase activity is blocked after staining by washing the cells twice with PBS and once with 1M KH_2PO_4. Glass cover slips are mounted with gel mount (Biomeda). The cells are examined under a fluorescence microscope using green light for excitation. Stained SKBR3 cells show intense red cell surface fluorescence.

8.2 Detection of c-erbB-2 protein over-expression in immunoblots using Fv(FRP5)-phoA: Proteins from total cell lysates of SKBR3 cells over-expressing c-erbB-2 protein are separated by SDS-PAGE and blotted onto PVDF membrane (Millipore). For preparation of extracts and immunoblotting technique see Example 1.3.2. Free binding sites of the membrane are blocked by incubation for 1 h at room temperature in a solution containing 10 mM Tris-HCl, pH 7.5, 0.9% NaCl, 0.05% Tween 20™ (BioRad), and 3% BSA. Pretreated Fv(FRP5)-phoA (see Example 7.2.) is diluted in blocking solution to a final concentration of 0.1 μg/ml. The membrane is incubated in the Fv(FRP5)-phoA solution for 1 h at room temperature and then washed three times for 5 min at room temperature in 10 mM Tris-HCl, pH 7.5, 0.9% NaCl, 0.05% Tween 20™ and once in 10 mM Tris-HCl, pH 7.5, 0.9% NaCl. For detection of bound Fv(FRP5)-phoA the membrane is incubated for 20 min at 37° C. in the Fast Red™ substrate solution described in Example 7.3 without levamisole. The reaction is stopped by washing the membrane twice in water. Fv(FRP5)-phoA specifically detects the 185 kD c-erbB-2 protein.

Example 9

Expression and Isolation of Fv(FRP5)-phoA from *E. coli*

9.1 Preparation of periplasmic extract: Plasmid pWW616 is transformed into the phoA negative *E. coli* strain CC118

according to standard procedures (Maniatis et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, 1982). A single colony is picked and grown overnight in LB medium containing 70 μg/ml ampicillin. The overnight culture is diluted 1:10 in fresh LB medium containing ampicillin and grown at 37° C. to an OD_{550} of 0.1. At this point expression of the Fv(FRP5)-phoA gene is induced by the addition of IPTG to a final concentration of 2 mM, and the cells are grown for an additional 1.5 to 2 h. The cells are harvested by centrifugation and treated with a mild osmotic shock which releases the periplasmatic proteins into the supernatant. The proteins are concentrated in an Amersham ultrafiltration unit through a YM10 membrane.

9.2 Preparation of an antigen affinity column: The c-erbB-2 protein is isolated from insect cells infected with a baculovirus vector expressing the c-erbB-2 extracellular domain by standard methods (V. A. Luckow & M. D. Summers, Biotechnology 6: 47–55, 1988). MAb FSP77 is coupled to CNBR-activated Sepharose 4B™ (Pharmacia) following the instructions of the manufacturer. The insect cell lysates are incubated with the coupled MAb FSP77 in a buffer containing 50 mM Tris-HCl, pH 7.5, 5 mM EGTA, 0.5% Triton X-100™, 150 mM NaCl for 2 h at 4° C. on a shaking platform. The beads are packed into a column and washed with pre-elution buffer consisting of 10 mM phosphate, pH 6.8, and 100 mM NaCl to remove non-specifically bound proteins. The c-erbB-2 protein is recovered from the column by treatment with a low pH elution buffer containing 100 mM glycine, pH 3.0, and 100 mM NaCl. The fractions from the column are collected into phosphate buffer, pH 8.0, in order to raise the pH. The c-erbB-2 extracellular domain is detected by running a part of each fraction on 8% SDS-PAGE gel, blotting onto PVDF membrane (Millipore) and treating the filter with MAb FSP77 followed by sheep anti-mouse IgG. Bound IgG is detected by ^{125}I-Protein-A treatment. The fractions containing the extracellular domain are pooled and the protein is coupled to CNBR-activated Sepharose 4B™ (Pharmacia) following the instructions of the manufacturer.

9.3 Isolation of Fv(FRP5)-phoA by affinity chromatography: The sepharose coupled to c-erbB-2 protein (Examle 9.2) is incubated for 2–4 h at 4° C. on a rocking platform with the periplasmic extract isolated as described in Example 9.1. The beads are packed into a column and washed with pre-elution buffer as in Example 9.2. The Fv(FRP5)-phoA protein is recovered by elution with the low pH elution buffer of Example 9.2. The fractions are monitored for the presence of the Fv(FRP5)-phoA by testing for phoA enzymatic activity using a standard protocol.

Example 10

Immunoassay for c-erbB-2 Protein in Tumors

10.1 Preparation of tumor sections: To determine the level of c-erbB-2 protein in tumors, tumor tissue is pretreated to give either frozen tumor sections or paraffin-embedded tumor sections. Tumor pieces are quick frozen, then cut with a cryostat, collected onto 1% gelatin-coated glass slides, and fixed with 4% paraformaldehyde. Following several washes with PBS, the tumor tissue sections are ready for staining. Alternatively, tumor pieces are placed in 4% paraformaldehyde for fixation, embedded in paraffin, then sections cut and collected onto polylysine-coated glass cover slips. To prepare the sections for staining, they are heated overnight at 56° C., dewaxed in xylene, stepwise rehydrated by washing in 95%, 70% and 35% ethanol and water, and washed in PBS.

10.2 Pretreatment of Fv(FRP5)-phoA: Since the dimer of the Fv(FRP5)-phoA as obtained from the *E. coli* periplasm does not bind optimally to the c-erbB-2 antigen, it is first monomerized. This is accomplished by treating the solution of Fv(FRP5)-phoA for 1 h at 37° C. with EGTA at a final concentration of 5 mM. This treatment chelates the Zn^{2+} ions which are important for maintaining the dimeric structure of Fv(FRP5)-phoA.

10.3 Staining of the tumor sections: Non-specific staining of the tumor sections prepared according to Example 10.1 is blocked by incubating the sections in PBS containing 3% BSA. The blocked sections are incubated for 1–2 h with pretreated Fv(FRP5)-phoA (Example 10.2) at a concentration of 1 μg/ml in a humidified chamber at room temperature. The sections are washed three times with PBS at room temperature. The bound Fv(FRP5)-phoA protein is detected using Fast Red™ as a substrate for the alkaline phosphatase. The staining solution consists of 300 μl naphthol AS-MX phosphate (Sigma, 13 mg/ml in dimethylformamide), 8 mg of levamisole (an inhibitor of endogenous alkaline phosphatase, Sigma), and 10 mg of Fast Red TR™ salt (Sigma) added to 9.7 ml of 100 mM Tris-HCl, pH 8.2, and 1 mM $ZnCl_2$. This mixture is prepared and filtered through a 0.45 μm filter immediately before use. $ZnCl_2$ is added to the staining solution to allow re-dimerization of the bound Fv(FRP5)-phoA protein and activation of the alkaline phosphatase. The tumor sections treated with Fv(FRP5)-phoA are incubated in the Fast Red™ staining solution for 15 min at room temperature. After staining the phosphatase activity is blocked by washing the cells twice with PBS and once with 1M KH_2PO_4. The glass cover slips are mounted with gel mount. The cells are examined under a fluorescence microscope using green light for excitation. Positively stained cells show an intense red cell surface fluorescence.

Alternatively, the tumor sections treated with the Fv(FRP5)-phoA protein may be stained with naphthol AS-BI phosphate (Sigma) and New Fuchsin™ (Sigma), or with 5-bromo-4-chloro-3-indolyl phosphate (BCIP, Sigma) and Nitro Blue Tetrazolium™ (Sigma). The stained sections can then be viewd with a regular light microscope.

Example 11

Cloning of Functional Heavy and Light Chain Rearrangements from the FWP51 Hybridoma Cell Line

Poly(A)-containing RNA isolated from FWP51 hybridoma cells as described in Example 3.3 provides the source for cDNA synthesis and subsequent amplification of V-region minigenes. cDNA synthesis and amplification of FWP51 heavy and light chain variable domain cDNA by polymerase chain reaction is carried out as described in Example 4. Amplification products of the expected size are purified from agarose gels and cloned into appropriate vectors. Functional rearrangements are identified by sequencing.

11.1 Subcloning of FWP51 heavy and light chain variable domain cDNA: Material amplified according to Example 4.3 is extracted with $CHCl_3$ and precipitated in the presence of 200 mM LiCl. To facilitate cloning, the FWP51 heavy chain variable domain cDNA is cleaved with restriction enzymes PstI and BstEII, the fragment purified by agarose gel electrophoresis, and ligated to PstI and BstEII digested pWW15 DNA. The FWP51 light chain variable domain cDNA is

cleaved with restriction enzymes PvuII and BglII, the fragment is purified by agarose gel electrophoresis, and ligated to PvuII and BglII digested pWW15 DNA (cf. Example 5). Ligation, transformation, and screening for the desired ligation products are carried out as described in Example 4.5. The following plasmids are obtained:

PCR product	Plasmid clones
H	pWW15-VH51-1
	pWW15-VH51-2
	pWW15-VH51-3
L	pWW15-VL51-1
	pWW15-VL51-2
	pWW15-VL51-3

11.2 Sequencing: Sequencing is done as described in Example 4.6.

Plasmids pWW15-VH51-1 (SEQ ID NO:8), pWW15-VH51-2, pWW15-VH51-3 contain identical functional FWP51 heavy chain variable domain inserts. Plasmids pWW15-VL51-1 (SEQ ID NO:9), pWW15-VL51-2, pWW15-VL51-3 contain identical functional FWP51 kappa light chain variable domain inserts. Plasmids pWW15-VH51-1 and pWW15-VL51-1 are used as a source for further subcloning steps.

Example 12

Construction of the MAb FWP51 Single Chain Gene

12.1 Assembly of the Fv fusion gene: Plasmid pWW15-VH51-1 is digested with PstI and BstEII and the 342 bp heavy chain variable domain fragment of FWP51 is isolated. It is cloned into PstI/BstEII digested pWW15-VL-51-1 yielding the plasmid pWW15-Fv51 (SEQ ID NO:10 and 11).

12.2 Mutation of the single-chain Fv(FWP51) gene: To allow gene fusion between the single-chain Fv(FWP51) encoding gene from pWW15-Fv51 and effector genes the stop coding at sequence position 729 to 731 in pWWFv15-51 (SEQ ID NO:10 and 11) is deleted as follows (see also Example 6.1): plasmid DNA of pWW15-Fv51 is digested with BstEII and BglII and the linker sequence and FWP51 light chain variable domain encoding fragment is isolated. In another digestion, pWW15-Fv51 is cleaved with BstEII and BclI. Thus, the large fragment containing vector sequences and the FWP51 heavy chain variable domain encoding sequence is isolated. The BstEII/BglII V_L fragment is now inserted into BstEII/BclI cleaved pWW15-Fv51 containing V_H. The resulting plasmid pWW15-Fv51-ORF is used as a source for the construction of Fv(FWP51)-effector fusion genes.

Example 13

Construction of Single-chain Fv-exotoxin A Fusion Gene Expression Plasmids

The MAb FRP5 and MAb FWP51 single-chain Fv genes are fused to a truncated bacterial toxin, exotoxin A (ETA) from *Pseudomonas aeruginosa*. These chimeric genes encode recombinant immunotoxins which selectively inhibit protein synthesis in c-erbB-2 expressing cells.

13.1 Mutation of the Exotoxin A gene of *Pseudomonas aeruginosa* PAK: For the construction of Fv-exotoxin A (Fv-ETA) fusion genes the ETA gene from *Pseudomonas aeruginosa* PAK is mutated to delete the original cell binding domain I at the N-terminus of the toxin and to generate a XbaI cleavage site at the former domain I/domain II boundary of the ETA coding region. Plasmid pMS150A (Lory et al., J. Bacteriol. 170: 714, 1988) is linearized by EcoRI cleavage. 20 ng of the linearized template DNA is used for a 100 µl PCR reaction carried out as described previously using the following oligonucleotides as primers 1 and 2.

1: 5'-CACGGAAGCTTAAGGAGATCTGCATGCT-TCTAGAGGGCGGCAGCCTGGCCGCGCTG-3'

2: 5'-GCGGATCGCTTCGCCCAGGT-3'

Following HindIII/SalI digestion of the PCR products, a 201 bp fragment is isolated from a 1.5% agarose gel and inserted into HindIII/SalI digested plasmid pUC18. Ligation is carried out as described above. Ligated DNA is transformed into *E.coli* XL1 Blue™ (Stratagene). Two recombinant plasmids are isolated and the insert DNA is sequenced as described above using pUC universal and reverse primers (Boehringer). One plasmid containing the expected product is designated pWW22 (SEQ ID NO:12) and used as a source for further subcloning steps. Plasmid pWW22 is cleaved with HindIII and SalI, the mutated ETA gene fragment is isolated, and inserted into the large fragment of HindIII/SalI digested plasmid pMS 150A containing pUC9 vector sequences and part of the ETA gene coding for the C-terminal half of the toxin. Thereby in the resulting plasmid pWW20 a truncated ETA gene coding for domains II and III of the toxin is created.

3.2 Assembly of single-chain Fv-ETA fusion genes: HindIII/XbaI single-chain Fv gene fragments suitable for the construction of Fv-ETA fusion genes are isolated from plasmid pWW53 (single-chain Fv FRP5), and plasmid pWW15-Fv51-ORF (single-chain Fv FWP51) and inserted into HindIII/XbaI digested pWW20. Ligation and transformation into *E.coli* XL1 Blue™ (Stratagene) are carried out as described above. The resulting plasmids pWW20-Fv5 (Fv(FRP5)-ETA) and pWW20-Fv51 (Fv(FWP51)-ETA) are used as a source for further subcloning steps.

13.3 Construction of single-chain Fv-exotoxin A fusion gene expression plasmids: For the expression of single-chain Fv-exotoxin A fusion genes in *E.coli* the expression plasmid pFLAG-1 (IBI Biochemicals) is used. The fusion-genes are fused in frame to the outer membrane protein A (ompA) signal sequence encoded by pFLAG-1. Plasmid DNA from pWW20-Fv5 and pWW20-Fv51 is digested with HindIII and blunt ends are created by Klenow fill-in as described in Example 4.5. Blunt ended DNA is digested with EcoRI and single-chain Fv-ETA gene fragments are isolated (Fv(FRP5)-ETA: 1916 bp, Fv(FWP51)-ETA: 1916 bp). pFLAG-1 plasmid DNA is digested with HindIII, blunt ends are created as described above, the resulting DNA fragment is isolated, and digested with EcoRI. Blunt-end/EcoR1 Fv-ETA fusion gene fragments are inserted into the modified pFLAG-1 plasmid DNA. Thereby Fv-ETA fragments are fused in frame to the ompA signal sequence of pFLAG-1 creating plasmids pWW215-5 for the expression of Fv(FRP5)-ETA (SEQ ID NO:13 and 14) and pWW215-5 for the expression of Fv(FWP51)-ETA (SEQ ID NO:15 and 16).

Example 14

Expression and Isolation of Fv(FRP5)-ETA and Fv(FWP51)-ETA from *E. coli*

14.1 Preparation of total lysates: Plasmids pWW215-5 and pWW215-51 are transformed into the *E.coli* strain

CC118 according to standard procedures (see Example 9.1). Single colonies are picked and grown overnight in LB medium containing 100 μg/ml ampicillin and 0.4% glucose. The overnight cultures are diluted 1:30 in fresh LB medium containing ampicillin and glucose and grown at 37° C. to an OD_{550} of 0.5. At this point expression of the Fv(FRP5)-ETA and Fv(FWP51)-ETA genes is induced by the addition of IPTG to a final concentration of 0.5 mM, and the cells are grown for an additional 30 min. The cells are harvested by centrifugation and lysed by sonication in PBS/1 mM $CaCl_2$. The lysates are cleared by ultracentrifugation at 25 000 g for 45 min at 4° C. The supernatants are collected.

14.2 Isolation of Fv(FRP5)-ETA and Fv(FWP51)-ETA by affinity chromatography: Cleared *E.coli* lysates containing the 66.4 kDa Fv(FRP5)-ETA or the 66.3 kDa Fv(FWP51)-ETA protein are passed through a M1 monoclonal antibody affinity column (IBI Biochemicals). The column is washed three times with PBS/1 mM $CaCl_2$. Bound Fv(FRP5)-ETA or Fv(FWP51)-ETA proteins are eluted with PBS/2 mM EDTA. The fractions are monitored for the presence of Fv-ETA proteins by SDS-PAGE and immunoblotting (see Example 1.3.2) using an anti-exotoxin A antiserum developed in rabbit.

Example 15

Selective Inhibition of Protein Synthesis in c-erbB-2 Expressing Cells With Fv(FRP5)-ETA and Fv(FWP51)-ETA

In vitro the recombinant immunotoxins Fv(FRP5)-ETA and Fv(FWP51)-ETA selectively inhibit protein synthesis and growth of cells expressing high levels of the human c-erbB-2 protein. The immunotoxins do not affect cells expressing no, or low levels of human c-erbB-2 protein.

15.1 Immunotoxin treatment of cell lines: Human breast and ovarian tumor cell lines SK-BR3, MDAMB-231, MDA-MB-453, HTB77, the mouse mammary epithelial cell line HC11, and HC11 cells transfected with the human c-erbB-2 cDNA are plated on 48 well tissue culture plates (Costar) at a density of 10^{5} cells/well. After 4 h the medium is removed and replaced by normal growth medium containing Fv(FRP5)-ETA or Fv(FWP51)-ETA at various concentrations ranging from 1 to 1000 ng/ml. The cells are incubated with toxin fusion proteins for 16 h.

15.2 ^{3}H-leucine labeling of cells: The immunotoxin-treated cells are washed twice and incubated in normal growth medium containing 4 μCi ^{3}H-leucin/ml for 4 h. The labeled cells are washed twice and ^{3}H-leucine labeled total proteins are harvested by TCA precipitation onto Whatman GFC filters. The rate of protein synthesis in immunotoxin-treated cells is determined in comparison to untreated control cells.

Example 16

Fv(FRP5)-ETA and MAbs FWP51 and FSP77 Inhibit the Growth of c-erbB-2 Expressing Cells in Nude Mice

The administration of Fv(FRP5)-ETA and the MAbs FWP51 and FSP77 to animals injected with c-erbB-2 expressing cells inhibits the tumor growth of these cells.

16.1 Nude mouse tumor model.: The NIH/3T3 mouse fibroblast cell line is transfected according to conventional, previously described methods (Graham & van der Eb, Virology 52: 456, 1973) with a plasmid expressing the point mutated, activated human c-erbB-2 protein (Masuko et al., Jpn. Cancer Res. 80: 10, 1989) and with the plasmid pSV2neo (Southern & Berg, J. Mol. Appl. Genet. 1:327, 1982) which encodes the gene for resistance to the drug G418. Transfected cells are selected 2 weeks in medium containing 500 ug/ml G418 (Geneticin, Gibco-BRL). Individual clones are selected and analyzed for the expression of the human c-erbB-2 protein using conventional protein blotting techniques (Towbin et al., Proc. Natl. Acad. Sci. USA 76: 4350, 1979). A clone expressing moderate levels of the point mutated, activated human c-erbB-2 protein (clone 3.7) is selected, and tested for growth in nude mice. 2–5×106 clone 3.7 cells (per animal) suspended in 0.2 ml PBS are subcutaneously injected into the flank of female Balb/c nude mice. The 3.7 cells injected at a dose of 2×106 cells rapidly form tumors in nude mice (control animals, cf. Example 16.2)

16.2 Immunotoxin treatment of animals: 2×106 clone 3.7 cells are injected subcutaneously into nude mice. The animals are treated continuously for a total of 7 days with the Fv(FRP5)-ETA. 200 μl of Fv(FRP5)-ETA (concentration 35 μg/ml in PBS) is placed in an osmotic pump (Alzet mini osmotic pump, Model 2001, Alza, Palo Alto, Calif., #94303-0802) which is implanted subcutaneously into the animals at the same time as the clone 3.7 cells are injected. The pump continuously releases Fv(FRP5)-ETA and delivers 1 μg/day for 7 days to each animal. In comparison with the control animals (cf. Example 16.1), the administration of Fv(FRP5)-ETA delays the onset of tumor formation.

16.3 MAb treatment of animals: 5×106 clone 3.7 cells are injected subcutaneously into nude mice. Starting on the same day as injection of clone 3.7 cells, the animals are treated daily, for a total of 10 days, with either MAb FWP51 or MAb FSP77 (MAb dose is 50 ug/200 ul BSS/day). The MAb is injected intraveneously in the tail vein of the mouse. Both antibodies delay the onset of tumor growth. Compared therewith, a synergistic effect in inhibiting tumor growth is observed on simultaneous administration of both antibodies MAb FWP51 and MAb FSP77.

DESCRIPTION OF THE SEQUENCES

SEQ ID NO:1 is the nucleotide sequence of pM716/1, which encodes the heavy chain variable domain of monoclonal antibody FRP5.

SEQ ID NO:2 is the nucleotide sequence of pM718/1, which encodes the kappa light chain variable domain of monoclonal antibody FRP5.

SEQ ID NO:3 is the nucleotide sequence of pWW19, which is used to link the heavy and light chain variable domains.

SEQ ID NO:4 is the nucleotide sequence of pWW52, which encodes the Fv heavy chain/light chain variable domain fusion protein binding to the growth factor receptor c-erbB-2.

SEQ ID NO:5 is the amino acid sequence of the Fv heavy chain/light chain variable domain fusion protein binding to the growth factor receptor c-erbB-2 encoded by pWW52.

SEQ ID NO:6 is the nucleotide sequence of pWW616, which encodes the Fv heavy chain/light chain variable domain and alkaline phosphatase fusion protein Fv(FRP5)-phoA binding to the growth factor receptor c-erbB-2.

SEQ ID NO:7 is the amino acid sequence of Fv heavy chain/light chain variable domain and alkaline phosphatase fusion protein Fv(FRP5)-phoA binding to the growth factor receptor c-erbB-2 encoded by pWW616.

SEQ ID NO:8 is the nucleotide sequence of pWW15-VH51-1, which encodes the heavy chain variable domain of monoclonal antibody FWP51.

SEQ ID NO:9 is the nucleotide sequence of pWW15-VL51-1, which encodes the light chain variable domain of monoclonal antibody FWP51.

SEQ ID NO:10 is the nucleotide sequence of pWW15-Fv51, which encodes single-chain Fv fusion gene comprising monoclonal antibody FWP51 heavy and kappa light chain variable domains.

SEQ ID NO:11 is the amino acid sequence of single-chain Fv fusion gene comprising monoclonal antibody FWP51 heavy and kappa light chain variable domains, encoded by pWW15-Fv51.

SEQ ID NO:12 is the nucleotide sequence of pWW22, which encodes part of the mutated exotoxin A gene from *Pseudomonas aeruginosa* PAK.

SEQ ID NO:13 is the nucleotide sequence of pWW215-5, which encodes Fv heavy chain/light chain variable domain and exotoxin A fusion protein Fv(FRP5)-ETA binding to the c-erbB-2 protein.

SEQ ID NO:14 is the amino acid sequence of the Fv heavy chain/light chain variable domain and exotoxin A fusion protein Fv(FRP5)-ETA binding to the c-erbB-2 protein encoded by pWW215-5.

SEQ ID NO:15 is the nucleotide sequence of pWW215-51, which encodes Fv heavy chain/light chain variable domain and exotoxin A fusion protein Fv(FWP51)-ETA binding to the c-erbB-2 protein.

SEQ ID NO:16 is the amino acid sequence for Fv heavy chain/light chain variable domain and exotoxin A fusion protein Fv(FWP51)-ETA binding to the c-erbB-2 protein, encoded by pWW215-51.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i i i) NUMBER OF SEQUENCES: 16

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 361 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: DNA

(i i i) HYPOTHETICAL: NO

(i v) ANTI-SENSE: NO

(v i) ORIGINAL SOURCE:
(A) ORGANISM: Mouse
(C) INDIVIDUAL ISOLATE: E. coli

(v i i) IMMEDIATE SOURCE:
(B) CLONE: pMZ16/1

(i x) FEATURE:
(A) NAME/KEY: primer_bind
(B) LOCATION: 6..27
(D) OTHER INFORMATION: /note="VH1BACK primer region"

(i x) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 95..328
(D) OTHER INFORMATION: /note="from 95 to 109 CDR1H; from 152 to 202 CDR2H; from 299 to 328 CDR3H"

(i x) FEATURE:
(A) NAME/KEY: primer_bind
(B) LOCATION: 329..361
(D) OTHER INFORMATION: /note="VH1FOR primer region"

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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GATCTCCTGC AAGGCCTCTG GGTATCCTTT CACAAACTAT GGAATGAACT GGGTGAAGCA     120
GGCTCCAGGA CAGGGTTTAA AGTGGATGGG CTGGATTAAC ACCTCCACTG GAGAGTCAAC     180
ATTTGCTGAT GACTTCAAGG GACGGTTTGA CTTCTCTTTG GAAACCTCTG CCAACACTGC     240
CTATTTGCAG ATCAACAACC TCAAAAGTGA AGACATGGCT ACATATTTCT GTGCAAGATG     300
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-continued

GGAGGTTTAC CACGGCTACG TTCCTTACTG GGGCCAAGGG ACCACGGTCA CCGTCTCCTC 360

A 361

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 407 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: DNA

(i i i) HYPOTHETICAL: NO

(i v) ANTI-SENSE: NO

(v i) ORIGINAL SOURCE:
(A) ORGANISM: Mouse
(C) INDIVIDUAL ISOLATE: E. coli

(v i i) IMMEDIATE SOURCE:
(B) CLONE: pMZ18/1

(i x) FEATURE:
(A) NAME/KEY: primer_bind
(B) LOCATION: 6..28
(D) OTHER INFORMATION: /note="MCK2 primer region"

(i x) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 98..319
(D) OTHER INFORMATION: /note="from 98 to 130 CDR1L; from 176 to 196 CDR2L; from 293 to 319 CDR3L"

(i x) FEATURE:
(A) NAME/KEY: primer_bind
(B) LOCATION: 374..404
(D) OTHER INFORMATION: /note="MCK2 primer region"

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:2:

TCTAGTCACT GGATGGTGGG AAGATGGAGA CATTGTGATG ACCCAGTCTC ACAAATTCCT 60

GTCCACTTCA GTAGGAGACA GGGTCAGCAT CACCTGCAAG GCCAGTCAGG ATGTGTATAA 120

TGCTGTTGCC TGGTATCAAC AGAAACCAGG ACAATCTCCT AAACTTCTGA TTTACTCCGC 180

ATCCTCCCGG TACACTGGAG TCCCTTCTCG CTTCACTGGC AGTGGCTCTG GGCCGGATTT 240

CACTTTCACC ATCAGCAGTG TGCAGGCTGA AGACCTGGCA GTTTATTTCT GTCAGCAACA 300

TTTTCGTACT CCATTCACGT TCGGCTCGGG GACAAAATTG GAAATAAAAC GGGCTGATGC 360

TGCACCAACT GTATCCATCT TCCCACCATC CAGTGACTAG AACTAGA 407

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 175 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: DNA

(i i i) HYPOTHETICAL: NO

(i v) ANTI-SENSE: NO

(v i) ORIGINAL SOURCE:
(A) ORGANISM: Synthetic
(C) INDIVIDUAL ISOLATE: E. coli

(v i i) IMMEDIATE SOURCE:
(B) CLONE: pWW19

-continued

(i x) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 30..117
(D) OTHER INFORMATION: /note="30-35 PstI site;38-44 BstEII site for subcloning of heavy chain var domain;54-98 coding seq of (GlyGlyGlyGlySer)3; 105-110 PvuII site; 112-117 BglII site

(i x) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 120..125
(D) OTHER INFORMATION: /note="BclI site for subcloning of light chain variable domain"

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:3:

```
AAGCTTGCAT GCAAGCTTCT CAGGTACAAC TGCAGGAGGT CACCGTTTCC TCTGGCGGTG      60

GCGGTTCTGG TGGCGGTGGC TCCGGCGGTG GCGGTTCTGA CATCCAGCTG GAGATCTAGC     120

TGATCAAAGC TCTAGAGGAT CCCCGGGTAC CGAGCTCGAA TTCACTGGCC GTCGT          175
```

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 748 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: DNA

(i i i) HYPOTHETICAL: NO

(i v) ANTI-SENSE: NO

(v i) ORIGINAL SOURCE:
(A) ORGANISM: Mouse
(C) INDIVIDUAL ISOLATE: E. coli

(v i i) IMMEDIATE SOURCE:
(B) CLONE: pWW52

(i x) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 6..728

(i x) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 1..701
(D) OTHER INFORMATION: /note="1-8 synthetic spacer;9-365 FRP5 heavy chain var. domain;99-113 CDR1H;156-206 CDR2H;303- 332 CDR3H;366-410 15 aa linker seq;411-728 FRP5 light chain var dom;480-512 CDR1L;558-578 CDR2L; 675-701 CDR3L

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:4:

```
AAGCT TCT CAG GTA CAA CTG CAG CAG TCT GGA CCT GAA CTG AAG AAG          47
      Ser Gln Val Glu Leu Gln Gln Ser Gly Pro Glu Leu Lys Lys
  1               5                  10

CCT GGA GAG ACA GTC AAG ATC TCC TGC AAG GCC TCT GGG TAT CCT TTC        95
Pro Gly Glu Thr Val Lys Ile Ser Cys Lys Ala Ser Gly Tyr Pro Phe
 15                  20                  25                  30

ACA AAC TAT GGA ATG AAC TGG GTG AAG CAG GCT CCA GGA CAG GGT TTA       143
Thr Asn Tyr Gly Met Asn Trp Val Lys Gln Ala Pro Gly Gln Gly Leu
      35                  40                  45

AAG TGG ATG GGC TGG ATT AAC ACT TCC ACT GGA GAG TCA ACA TTT GCT       191
Lys Trp Met Gly Trp Ile Asn Thr Ser Thr Gly Glu Ser Thr Phe Ala
      50                  55                  60

GAT GAC TTC AAG GGA CGG TTT GAC TTC TCT TTG GAA ACC TCT GCC AAC       239
Asp Asp Phe Lys Gly Arg Phe Asp Phe Ser Leu Glu Thr Ser Ala Asn
  65                  70                  75

ACT GCC TAT TTG CAG ATC AAC AAC CTC AAA AGT GAA GAC ATG GCT ACA       287
```

-continued

```
Thr Ala Tyr Leu Gln Ile Asn Asn Leu Lys Ser Glu Asp Met Ala Thr
 80                  85                  90

TAT TTC TGT GCA AGA TGG GAG GTT TAC CAC GGC TAC GTT CCT TAC TGG      335
Tyr Phe Cys Ala Arg Trp Glu Val Tyr His Gly Tyr Val Pro Tyr Trp
 95                 100                 105                 110

GGC CAA GGG ACC ACG GTC ACC GTT TCC TCT GGC GGT GGC GGT TCT GGT      383
Gly Gln Gly Thr Thr Val Thr Val Ser Ser Gly Gly Gly Gly Ser Gly
            115                 120                 125

GGC GGT GGC TCC GGC GGT GGC GGT GGT TCT GAC ATC CAG CTG ACC CAG TCT  431
Gly Gly Gly Ser Gly Gly Gly Gly Ser Asp Ile Gln Leu Thr Gln Ser
        130                 135                 140

CAC AAA TTC CTG TCC ACT TCA GTA GGA GAC AGG GTC AGC ATC ACC TGC      479
His Lys Phe Leu Ser Thr Ser Val Gly Asp Arg Val Ser Ile Thr Cys
145                 150                 155

AAG GCC AGT CAG GAT GTG TAT AAT GCT GTT GCC TGG TAT CAA CAG AAA      527
Lys Ala Ser Gln Asp Val Tyr Asn Ala Val Ala Trp Tyr Gln Gln Lys
            160                 165                 170

CCA GGA CAA TCT CCT AAA CTT CTG ATT TAC TCG GCA TCC TCC CGG TAC      575
Pro Gly Gln Ser Pro Lys Leu Leu Ile Tyr Ser Ala Ser Ser Arg Tyr
175                 180                 185                 190

ACT GGA GTC CCT TCT CGC TTC ACT GGC AGT GGC TCT GGG CCG GAT TTC      623
Thr Gly Val Pro Ser Arg Phe Thr Gly Ser Gly Ser Gly Pro Asp Phe
            195                 200                 205

ACT TTC ACC ATC AGC AGT GTG CAG GCT GAA GAC CTG GCA GTT TAT TTC      671
Thr Phe Thr Ile Ser Ser Val Gln Ala Glu Asp Leu Ala Val Tyr Phe
        210                 215                 220

TGT CAG CAA CAT TTT CGT ACT CCA TTC ACG TTC GGC TCG GGG ACA AAA      719
Cys Gln Gln His Phe Arg Thr Pro Phe Thr Phe Gly Ser Gly Thr Lys
225                 230                 235

TTG GAG ATC TAGCTGATCA AAGCTCTAGA                                   748
Leu Glu Ile
    240
```

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 241 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: protein

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:5:

```
Ser Gln Val Gln Leu Glu Gln Ser Gly Pro Glu Leu Lys Lys Pro Gly
 1               5                  10                  15

Glu Thr Val Lys Ile Ser Cys Lys Ala Ser Gly Tyr Pro Phe Thr Asn
            20                  25                  30

Tyr Gly Met Asn Trp Val Lys Gln Ala Pro Gly Gln Gly Leu Lys Trp
        35                  40                  45

Met Gly Trp Ile Asn Thr Ser Thr Gly Glu Ser Thr Phe Ala Asp Asp
    50                  55                  60

Phe Lys Gly Arg Phe Asp Phe Ser Leu Glu Thr Ser Ala Asn Thr Ala
65                  70                  75                  80

Tyr Leu Gln Ile Asn Asn Leu Lys Ser Glu Asp Met Ala Thr Tyr Phe
                85                  90                  95

Cys Ala Arg Trp Glu Val Tyr His Gly Tyr Val Pro Tyr Trp Gly Gln
            100                 105                 110

Gly Thr Thr Val Thr Val Ser Ser Gly Gly Gly Gly Ser Gly Gly Gly
        115                 120                 125

Gly Ser Gly Gly Gly Gly Ser Asp Ile Gln Leu Thr Gln Ser His Lys
```

-continued

```
     130                 135                 140

Phe Leu Ser Thr Ser Val Gly Asp Arg Val Ser Ile Thr Cys Lys Ala
145                 150                 155                 160

Ser Glu Asp Val Tyr Asn Ala Val Ala Trp Tyr Gln Gln Lys Pro Gly
     165                 170                 175

Glu Ser Pro Lys Leu Leu Ile Tyr Ser Ala Ser Ser Arg Tyr Thr Gly
       180                 185                 190

Val Pro Ser Arg Phe Thr Gly Ser Gly Ser Gly Pro Asp Phe Thr Phe
   195                 200                 205

Thr Ile Ser Ser Val Gln Ala Glu Asp Leu Ala Val Tyr Phe Cys Gln
     210                 215                 220

Gln His Phe Arg Thr Pro Phe Thr Phe Gly Ser Gly Thr Lys Leu Glu
225                 230                 235                 240

Ile
```

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 2233 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: DNA

(i i i) HYPOTHETICAL: NO

(i v) ANTI-SENSE: NO

(v i) ORIGINAL SOURCE:
(A) ORGANISM: Mouse and E. coli
(C) INDIVIDUAL ISOLATE: E. coli

(v i i) IMMEDIATE SOURCE:
(B) CLONE: pWW616

(i x) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 23..2155
(D) OTHER INFORMATION: /note="89-445 FRP5 heavy chain vardomain; 446-490 15 aa linker sequence; 491-814 FRP5 light chain vardomain; 815-2155 coding region of phoA

(i x) FEATURE:
(A) NAME/KEY: 5'UTR
(B) LOCATION: 1..22
(D) OTHER INFORMATION: /function="ompA 5'non-coding region"

(i x) FEATURE:
(A) NAME/KEY: sig_peptide
(B) LOCATION: 23..85
(D) OTHER INFORMATION: /note="ompA signal peptide"

(i x) FEATURE:
(A) NAME/KEY: 3'UTR
(B) LOCATION: 2156..2233
(D) OTHER INFORMATION: /function="phoA 3'non-coding region"

(i x) FEATURE:
(A) NAME/KEY: mat_peptide
(B) LOCATION: 86..2155

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:6:

```
TCTAGATAAC GAGGCGCAAA AA ATG AAA AAG ACA GCT ATC GCG ATT GCA GTG      52
          Met Lys Lys Thr Ala Ile Ala Ile Ala Val
          -21 -20                 -15

GCA CTG GCT GGT TTC GCT ACC GTA GCG CAA GCT TCT CAG GTA CAA CTG      100
```

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```
Ala Leu Ala Gly Phe Ala Thr Val Ala Gln Ala Ser Gln Val Gln Leu
-10                 -5                  1                   5

CAG CAG TCT GGA CCT GAA CTG AAG AAG CCT GGA GAG ACA GTC AAG ATC   148
Gln Gln Ser Gly Pro Glu Leu Lys Lys Pro Gly Glu Thr Val Lys Ile
10                  15                  20

TCC TGC AAG GCC TCT GGG TAT CCT TTC ACA AAC TAT GGA ATG AAC TGG   196
Ser Cys Lys Ala Ser Gly Tyr Pro Phe Thr Asn Tyr Gly Met Asn Trp
25                  30                  35

GTG AAG CAG GCT CCA GGA CAG GGT TTA AAG TGG ATG GGC TGG ATT AAC   244
Val Lys Gln Ala Pro Gly Gln Gly Leu Lys Trp Met Gly Trp Ile Asn
40                  45                  50

ACC TCC ACT GGA GAG TCA ACA TTT GCT GAT GAC TTC AAG GGA CGG TTT   292
Thr Ser Thr Gly Glu Ser Thr Phe Ala Asp Asp Phe Lys Gly Arg Phe
55                  60                  65

GAC TTC TCT TTG GAA ACC TCT GCC AAC ACT GCC TAT TTG CAG ATC AAC   340
Asp Phe Ser Leu Glu Thr Ser Ala Asn Thr Ala Tyr Leu Gln Ile Asn
70                  75                  80                  85

AAC CTC AAA AGT GAA GAC ATG GCT ACA TAT TTC TGT GCA AGA TGG GAG   388
Asn Leu Lys Ser Glu Asp Met Ala Thr Tyr Phe Cys Ala Arg Trp Glu
90                  95                  100

GTT TAC CAC GGC TAC GTT CCT TAC TGG GGC CAA GGG ACC ACG GTC ACC   436
Val Tyr His Gly Tyr Val Pro Tyr Trp Gly Gln Gly Thr Thr Val Thr
105                 110                 115

GTT TCC TCT GGC GGT GGC GGT TCT GGT GGC GGT GGC TCC GGC GGT GGC   484
Val Ser Ser Gly Gly Gly Gly Ser Gly Gly Gly Gly Ser Gly Gly Gly
120                 125                 130

GGT TCT GAC ATC CAG CTG ACC CAG TCT CAC AAA TTC CTG TCC ACT TCA   532
Gly Ser Asp Ile Gln Leu Thr Gln Ser His Lys Phe Leu Ser Thr Ser
135                 140                 145

GTA GGA GAC AGG GTC AGC ATC ACC TGC AAG GCC AGT CAG GAT GTG TAT   580
Val Gly Asp Arg Val Ser Ile Thr Cys Lys Ala Ser Gln Asp Val Tyr
150                 155                 160                 165

AAT GCT GTT GCC TGG TAT CAA CAG AAA CCA GGA CAA TCT CCT AAA CTT   628
Asn Ala Val Ala Trp Tyr Gln Gln Lys Pro Gly Gln Ser Pro Lys Leu
170                 175                 180

CTG ATT TAC TCG GCA TCC TCC CGG TAC ACT GGA GTC CCT TCT CGC TTC   676
Leu Ile Tyr Ser Ala Ser Ser Arg Tyr Thr Gly Val Pro Ser Arg Phe
185                 190                 195

ACT GGC AGT GGC TCT GGG CCG GAT TTC ACT TTC ACC ATC AGC AGT GTG   724
Thr Gly Ser Gly Ser Gly Pro Asp Phe Thr Phe Thr Ile Ser Ser Val
200                 205                 210

CAG GCT GAA GAC CTG GCA GTT TAT TTC TGT CAG CAA CAT TTT CGT ACT   772
Gln Ala Glu Asp Leu Ala Val Tyr Phe Cys Gln Gln His Phe Arg Thr
215                 220                 225

CCA TTC ACG TTC GGC TCG GGG ACA AAA TTG GAG ATC AAA GCT CTA GAG   820
Pro Phe Thr Phe Gly Ser Gly Thr Lys Leu Glu Ile Lys Ala Leu Glu
230                 235                 240                 245

CCT GTT CTG GAA AAC CGG GCT GCT CAG GGC GAT ATT ACT GCA CCC GGC   868
Pro Val Leu Glu Asn Arg Ala Ala Gln Gly Asp Ile Thr Ala Pro Gly
250                 255                 260

GGT GCT CGC CGT TTA ACG GGT GAT CAG ACT GCC GCT CTG CGT GAT TCT   916
Gly Ala Arg Arg Leu Thr Gly Asp Gln Thr Ala Ala Leu Arg Asp Ser
265                 270                 275

CTT AGC GAT AAA CCT GCA AAA AAT ATT ATT TTG CTG ATT GGC GAT GGG   964
Leu Ser Asp Lys Pro Ala Lys Asn Ile Ile Leu Leu Ile Gly Asp Gly
280                 285                 290

ATG GGG GAC TCG GAA ATT ACT GCC GCA CGT AAT TAT GCC GAA GGT GCG   1012
Met Gly Asp Ser Glu Ile Thr Ala Ala Arg Asn Tyr Ala Glu Gly Ala
295                 300                 305

GGC GGC TTT TTT AAA GGT ATA GAT GCC TTA CCG CTT ACC GGG CAA TAC   1060
```

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```
Gly Gly Phe Phe Lys Gly Ile Asp Ala Leu Pro Leu Thr Gly Gln Tyr
310                 315                 320                 325

ACT CAC TAT GCG CTG AAT AAA AAA ACC GGC AAA CCG GAC TAC GTC ACC      1108
Thr His Tyr Ala Leu Asn Lys Lys Thr Gly Lys Pro Asp Tyr Val Thr
     330                 335                 340

GAC TCG GCT GCA TCA GCA ACC GCC TGG TCA ACC GGT GTC AAA ACC TAT      1156
Asp Ser Ala Ala Ser Ala Thr Ala Trp Ser Thr Gly Val Lys Thr Tyr
345                 350                 355

AAC GGC GCG CTG GGC GTC GAT ATT CAC GAA AAA GAT CAC CCA ACG ATT      1204
Asn Gly Ala Leu Gly Val Asp Ile His Glu Lys Asp His Pro Thr Ile
360                 365                 370

CTG GAA ATG GCA AAA GCC GCA GGT CTG GCG ACC GGT AAC GTT TCT ACC      1252
Leu Glu Met Ala Lys Ala Ala Gly Leu Ala Thr Gly Asn Val Ser Thr
375                 380                 385

GCA GAG TTG CAG GAT GCC ACG CCC GCT GCG CTG GTG GCA CAT GTG ACC      1300
Ala Glu Leu Gln Asp Ala Thr Pro Ala Ala Leu Val Ala His Val Thr
390                 395                 400                 405

TCG CGC AAA TGC TAC GGT CCG AGC GCG ACC AGT GAA AAA TGT CCG GGT      1348
Ser Arg Lys Cys Tyr Gly Pro Ser Ala Thr Ser Glu Lys Cys Pro Gly
410                 415                 420

AAC GCT CTG GAA AAA GGC GGA AAA GGA TCG ATT ACC GAA CAG CTG CTT      1396
Asn Ala Leu Glu Lys Gly Gly Lys Gly Ser Ile Thr Glu Gln Leu Leu
425                 430                 435

AAC GCT CGT GCC GAC GTT ACG CTT GGC GGC GGC GCA AAA ACC TTT GCT      1444
Asn Ala Arg Ala Asp Val Thr Leu Gly Gly Gly Ala Lys Thr Phe Ala
440                 445                 450

GAA ACG GCA ACC GCT GGT GAA TGG CAG GGA AAA ACG CTG CGT GAA CAG      1492
Glu Thr Ala Thr Ala Gly Glu Trp Gln Gly Lys Thr Leu Arg Glu Gln
455                 460                 465

GCA CAG GCG CGT GGT TAT CAG TTG GTG AGC GAT GCT GCC TCA CTG AAT      1540
Ala Gln Ala Arg Gly Tyr Gln Leu Val Ser Asp Ala Ala Ser Leu Asn
470                 475                 480                 485

TCG GTG ACG GAA GCG AAT CAG CAA AAA CCC CTG CTT GGC CTG TTT GCT      1588
Ser Val Thr Glu Ala Asn Gln Gln Lys Pro Leu Leu Gly Leu Phe Ala
490                 495                 500

GAC GGC AAT ATG CCA GTG CGC TGG CTA GGA CCG AAA GCA ACG TAC CAT      1636
Asp Gly Asn Met Pro Val Arg Trp Leu Gly Pro Lys Ala Thr Tyr His
505                 510                 515

GGC AAT ATC GAT AAG CCC GCA GTC ACC TGT ACG CCA AAT CCG CAA CGT      1684
Gly Asn Ile Asp Lys Pro Ala Val Thr Cys Thr Pro Asn Pro Gln Arg
520                 525                 530

AAT GAC AGT GTA CCA ACC CTG GCG CAG ATG ACC GAC AAA GCC ATT GAA      1732
Asn Asp Ser Val Pro Thr Leu Ala Gln Met Thr Asp Lys Ala Ile Glu
535                 540                 545

TTG TTG AGT AAA AAT GAG AAA GGC TTT TTC CTG CAA GTT GAA GGT GCG      1780
Leu Leu Ser Lys Asn Glu Lys Gly Phe Phe Leu Gln Val Glu Gly Ala
550                 555                 560                 565

TCA ATC GAT AAA CAG GAT CAT GCT GCG AAT CCT TGT GGG CAA ATT GGC      1828
Ser Ile Asp Lys Gln Asp His Ala Ala Asn Pro Cys Gly Gln Ile Gly
570                 575                 580

GAG ACG GTC GAT CTC GAT GAA GCC GTA CAA CGG GCG CTG GAA TTC GCT      1876
Glu Thr Val Asp Leu Asp Glu Ala Val Gln Arg Ala Leu Glu Phe Ala
585                 590                 595

AAA AAG GAG GGT AAC ACG CTG GTC ATA GTC ACC GCT GAT CAC GCC CAC      1924
Lys Lys Glu Gly Asn Thr Leu Val Ile Val Thr Ala Asp His Ala His
600                 605                 610

GCC AGC CAG ATT GTT GCG CCG GAT ACC AAA GCT CCG GGC CTC ACC CAG      1972
Ala Ser Gln Ile Val Ala Pro Asp Thr Lys Ala Pro Gly Leu Thr Gln
615                 620                 625

GCG CTA AAT ACC AAA GAT GGC GCA GTG ATG GTG ATG AGT TAC GGG AAC      2020
```

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```
Ala Leu Asn Thr Lys Asp Gly Ala Val Met Val Met Ser Tyr Gly Asn
630                 635                 640                 645

TCC GAA GAG GAT TCA CAA GAA CAT ACC GGC AGT CAG TTG CGT ATT GCG      2068
Ser Glu Glu Asp Ser Gln Glu His Thr Gly Ser Gln Leu Arg Ile Ala
     650                 655                 660

GCG TAT GGC CCG CAT GCC GCC AAT GTT GTT GGA CTG ACC GAC CAG ACC      2116
Ala Tyr Gly Pro His Ala Ala Asn Val Val Gly Leu Thr Asp Gln Thr
         665                 670                 675

GAT CTC TTC TAC ACC ATG AAA GCC GCT CTG GGG CTG AAA TAAAACCGCG       2165
Asp Leu Phe Tyr Thr Met Lys Ala Ala Leu Gly Leu Lys
  680                 685                 690

CCCGGCAGTG AATTTTCGCT GCCGGGTGGT TTTTTTGCTG TTAGCAACCA GACTTAATGG    2225

CAGAGCTC                                                             2233
```

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 711 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: protein

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:7:

```
Met Lys Lys Thr Ala Ile Ala Ile Ala Val Ala Leu Ala Gly Phe Ala
-21  -20                 -15                 -10

Thr Val Ala Glu Ala Ser Gln Val Gln Leu Gln Gln Ser Gly Pro Glu
 -5                   1                   5                  10

Leu Lys Lys Pro Gly Glu Thr Val Lys Ile Ser Cys Lys Ala Ser Gly
          15                  20                  25

Tyr Pro Phe Thr Asn Tyr Gly Met Asn Trp Val Lys Gln Ala Pro Gly
      30                  35                  40

Gln Gly Leu Lys Trp Met Gly Trp Ile Asn Thr Ser Thr Gly Glu Ser
          45                  50                  55

Thr Phe Ala Asp Asp Phe Lys Gly Arg Phe Asp Phe Ser Leu Glu Thr
  60                  65                  70                  75

Ser Ala Asn Thr Ala Tyr Leu Gln Ile Asn Asn Leu Lys Ser Glu Asp
          80                  85                  90

Met Ala Thr Tyr Phe Cys Ala Arg Trp Glu Val Tyr His Gly Tyr Val
          95                 100                 105

Pro Tyr Trp Gly Gln Gly Thr Thr Val Thr Val Ser Ser Gly Gly Gly
     110                 115                 120

Gly Ser Gly Gly Gly Gly Ser Gly Gly Gly Gly Ser Asp Ile Gln Leu
     125                 130                 135

Thr Gln Ser His Lys Phe Leu Ser Thr Ser Val Gly Asp Arg Val Ser
140                 145                 150                 155

Ile Thr Cys Lys Ala Ser Gln Asp Val Tyr Asn Ala Val Ala Trp Tyr
          160                 165                 170

Gln Gln Lys Pro Gly Gln Ser Pro Lys Leu Leu Ile Tyr Ser Ala Ser
          175                 180                 185

Ser Arg Tyr Thr Gly Val Pro Ser Arg Phe Thr Gly Ser Gly Ser Gly
     190                 195                 200

Pro Asp Phe Thr Phe Thr Ile Ser Ser Val Gln Ala Glu Asp Leu Ala
     205                 210                 215

Val Tyr Phe Cys Gln Gln His Phe Arg Thr Pro Phe Thr Phe Gly Ser
220                 225                 230                 235

Gly Thr Lys Leu Glu Ile Lys Ala Leu Glu Pro Val Leu Glu Asn Arg
```

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```
240                 245                 250

Ala Ala Gln Gly Asp Ile Thr Ala Pro Gly Gly Ala Arg Arg Leu Thr
        255                 260                 265

Gly Asp Gln Thr Ala Ala Leu Arg Asp Ser Leu Ser Asp Lys Pro Ala
    270                 275                 280

Lys Asn Ile Ile Leu Leu Ile Gly Asp Gly Met Gly Asp Ser Glu Ile
        285                 290                 295

Thr Ala Ala Arg Asn Tyr Ala Glu Gly Ala Gly Gly Phe Phe Lys Gly
300                 305                 310                 315

Ile Asp Ala Leu Pro Leu Thr Gly Gln Tyr Thr His Tyr Ala Leu Asn
        320                 325                 330

Lys Lys Thr Gly Lys Pro Asp Tyr Val Thr Asp Ser Ala Ala Ser Ala
        335                 340                 345

Thr Ala Trp Ser Thr Gly Val Lys Thr Tyr Asn Gly Ala Leu Gly Val
    350                 355                 360

Asp Ile His Glu Lys Asp His Pro Thr Ile Leu Glu Met Ala Lys Ala
        365                 370                 375

Ala Gly Leu Ala Thr Gly Asn Val Ser Thr Ala Glu Leu Gln Asp Ala
380                 385                 390                 395

Thr Pro Ala Ala Leu Val Ala His Val Thr Ser Arg Lys Cys Tyr Gly
        400                 405                 410

Pro Ser Ala Thr Ser Glu Lys Cys Pro Gly Asn Ala Leu Glu Lys Gly
        415                 420                 425

Gly Lys Gly Ser Ile Thr Glu Gln Leu Leu Asn Ala Arg Ala Asp Val
    430                 435                 440

Thr Leu Gly Gly Gly Ala Lys Thr Phe Ala Glu Thr Ala Thr Ala Gly
        445                 450                 455

Glu Trp Gln Gly Lys Thr Leu Arg Glu Gln Ala Gln Ala Arg Gly Tyr
460                 465                 470                 475

Gln Leu Val Ser Asp Ala Ala Ser Leu Asn Ser Val Thr Glu Ala Asn
        480                 485                 490

Gln Gln Lys Pro Leu Leu Gly Leu Phe Ala Asp Gly Asn Met Pro Val
        495                 500                 505

Arg Trp Leu Gly Pro Lys Ala Thr Tyr His Gly Asn Ile Asp Lys Pro
    510                 515                 520

Ala Val Thr Cys Thr Pro Asn Pro Gln Arg Asn Asp Ser Val Pro Thr
        525                 530                 535

Leu Ala Gln Met Thr Asp Lys Ala Ile Glu Leu Leu Ser Lys Asn Glu
540                 545                 550                 555

Lys Gly Phe Phe Leu Gln Val Glu Gly Ala Ser Ile Asp Lys Gln Asp
        560                 565                 570

His Ala Ala Asn Pro Cys Gly Gln Ile Gly Glu Thr Val Asp Leu Asp
        575                 580                 585

Glu Ala Val Gln Arg Ala Leu Glu Phe Ala Lys Lys Glu Gly Asn Thr
    590                 595                 600

Leu Val Ile Val Thr Ala Asp His Ala His Ala Ser Gln Ile Val Ala
        605                 610                 615

Pro Asp Thr Lys Ala Pro Gly Leu Thr Gln Ala Leu Asn Thr Lys Asp
620                 625                 630                 635

Gly Ala Val Met Val Met Ser Tyr Gly Asn Ser Glu Glu Asp Ser Gln
        640                 645                 650

Glu His Thr Gly Ser Gln Leu Arg Ile Ala Ala Tyr Gly Pro His Ala
        655                 660                 665
```

-continued

```
Ala Asn Val Val Gly Leu Thr Asp Glu Thr Asp Leu Phe Tyr Thr Met
 670                 675                 680

Lys Ala Ala Leu Gly Leu Lys
 685                 690
```

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 342 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: DNA

(i i i) HYPOTHETICAL: NO

(i v) ANTI-SENSE: NO

(v i) ORIGINAL SOURCE:
(A) ORGANISM: Mouse
(C) INDIVIDUAL ISOLATE: E. coli

(v i i) IMMEDIATE SOURCE:
(B) CLONE: pWW15-VH51-1

(i x) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 1..342
(D) OTHER INFORMATION: /note="1-14 partial seq. of VH1BACK primer region; 82-96 CDR1H; 139-189 CDR2H; 286-318 CDR3H; 317-342 partial seq. of VH1FOR primer region

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:8:

```
CTGCAGCAGT CTGGGGCTGA GCTGGTGAGG CCTGGGACTT CAGTGAAGCT GTCCTGCAAG   60
GCTTCTGATT ACACCTTCAC CAGCTACTGG ATGAACTGGG TGAAGCAGAG GCCTGGACAA  120
GGCCTTGAAT GGATTGGTAT GATTGATCCT TCAGACAGTG AAACTCAATA CAATCAAATG  180
TTCAAGGACA AGGCCGCATT GACTGTAGAC AAGTCCTCCA ATACAGCCTA CATGCAACTC  240
AGCAGCCTGA CATCTGAGGA CTCTGCGGTC TATTACTGTG CAAAAGGGGG GGCCTCTGGG  300
GACTGGTACT TCGATGTCTG GGGCCAAGGG ACCACGGTCA CC                     342
```

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 310 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: DNA

(i i i) HYPOTHETICAL: NO

(i v) ANTI-SENSE: NO

(v i) ORIGINAL SOURCE:
(A) ORGANISM: Mouse
(C) INDIVIDUAL ISOLATE: E. coli

(v i i) IMMEDIATE SOURCE:
(B) CLONE: pWW15-VL51-1

(i x) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 1..310
(D) OTHER INFORMATION: /note="1-18 partial seq. of VK1BACK primer region; 64-96 CDR1L; 142-162 CDR2L; 259-282 CDR3L; 292-310 partial seq. of VK1FOR primer region

-continued

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:9:

```
CAGCTGACCC AGTCTCCATC CTCACTGTCT GCATCTCTGG GAGGCGAAGT CACCATCACT      60

TGCAAGGCAA GCCAAGACAT TAAGAAGTAT ATAGCTTGGT ACCAACACAA GCCTGGAAAA     120

AGTCCTCGGC TACTCATACA CTACACATCT GTATTACAGC CAGGCATCCC ATCCAGGTTC     180

AGTGGAAGTG GGTCTGGGAG AGATTATTCC TTCAGCATCC ACAACCTGGA GCCTGAAGAT     240

ATTGCAACTT ATTATTGTCT ACATTATGAT TATCTGTACA CGTTCGGAGG GGGCACCAAG     300

CTGGAGATCT                                                            310
```

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 748 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: DNA

(i i i) HYPOTHETICAL: NO

(i v) ANTI-SENSE: NO

(v i) ORIGINAL SOURCE:
(A) ORGANISM: Mouse
(C) INDIVIDUAL ISOLATE: E. coli

(v i i) IMMEDIATE SOURCE:
(B) CLONE: pWW15-Fv51

(i x) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 6..728
(D) OTHER INFORMATION: /note="1-8 synthetic spacer;9-368 FWP51 Heavy ChainVar.Dom.;99-113 CDR1H;156-206 CDR2H;303- 335 CDR3H;369-413 Syn.Spcr;414-728 FWP51 Light ChainVar.Dom;483-515 CDR1L;561-581 CDR2L; 729-748 Syn.Spcr.

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:10:

```
AAGCT TCT CAG GTA CAA CTG CAG CAG TCT GGG GCT GAG CTG GTG AGG          47
      Ser Gln Val Gln Leu Gln Gln Ser Gly Ala Glu Leu Val Arg
  1               5                  10

CCT GGG ACT TCA GTG AAG CTG TCC TGC AAG GCT TCT GAT TAC ACC TTC        95
Pro Gly Thr Ser Val Lys Leu Ser Cys Lys Ala Ser Asp Tyr Thr Phe
 15                  20                  25                  30

ACC AGC TAC TGG ATG AAC TGG GTG AAG CAG AGG CCT GGA CAA GGC CTT       143
Thr Ser Tyr Trp Met Asn Trp Val Lys Gln Arg Pro Gly Gln Gly Leu
       35                  40                  45

GAA TGG ATT GGT ATG ATT GAT CCT TCA GAC AGT GAA ACT CAA TAC AAT       191
Glu Trp Ile Gly Met Ile Asp Pro Ser Asp Ser Glu Thr Gln Tyr Asn
       50                  55                  60

CAA ATG TTC AAG GAC AAG GCC GCA TTG ACT GTA GAC AAG TCC TCC AAT       239
Gln Met Phe Lys Asp Lys Ala Ala Leu Thr Val Asp Lys Ser Ser Asn
   65                  70                  75

ACA GCC TAC ATG CAA CTC AGC AGC CTG ACA TCT GAG GAC TCT GCG GTC       287
Thr Ala Tyr Met Gln Leu Ser Ser Leu Thr Ser Glu Asp Ser Ala Val
       80                  85                  90

TAT TAC TGT GCA AAA GGG GGG GCC TCT GGG GAC TGG TAC TTC GAT GTC       335
Tyr Tyr Cys Ala Lys Gly Gly Ala Ser Gly Asp Trp Tyr Phe Asp Val
 95                  100                 105                 110

TGG GGC CAA GGG ACC ACG GTC ACC GTT TCC TCT GGC GGT GGC GGT TCT       383
Trp Gly Gln Gly Thr Thr Val Thr Val Ser Ser Gly Gly Gly Gly Ser
      115                 120                 125

GGT GGC GGT GGC TCC GGC GGT GGC GGT TCT GAC ATC CAG CTG ACC CAG       431
```

-continued

```
Gly Gly Gly Gly Ser Gly Gly Gly Gly Ser Asp Ile Gln Leu Thr Gln
        130                 135                 140

TCT CCA TCC TCA CTG TCT GCA TCT CTG GGA GGC GAA GTC ACC ATC ACT      479
Ser Pro Ser Ser Leu Ser Ala Ser Leu Gly Gly Glu Val Thr Ile Thr
  145                 150                 155

TGC AAG GCA AGC CAA GAC ATT AAG AAG TAT ATA GCT TGG TAC CAA CAC      527
Cys Lys Ala Ser Gln Asp Ile Lys Lys Tyr Ile Ala Trp Tyr Gln His
      160                 165                 170

AAG CCT GGA AAA AGT CCT CGG CTA CTC ATA CAC TAC ACA TCT GTA TTA      575
Lys Pro Gly Lys Ser Pro Arg Leu Leu Ile His Tyr Thr Ser Val Leu
175                 180                 185                 190

CAG CCA GGC ATC CCA TCC AGG TTC AGT GGA AGT GGG TCT GGG AGA GAT      623
Gln Pro Gly Ile Pro Ser Arg Phe Ser Gly Ser Gly Ser Gly Arg Asp
      195                 200                 205

TAT TCC TTC AGC ATC CAC AAC CTG GAG CCT GAA GAT ATT GCA ACT TAT      671
Tyr Ser Phe Ser Ile His Asn Leu Glu Pro Glu Asp Ile Ala Thr Tyr
      210                 215                 220

TAT TGT CTA CAT TAT GAT TAT CTG TAC ACG TTC GGA GGG GGC ACC AAG      719
Tyr Cys Leu His Tyr Asp Tyr Leu Tyr Thr Phe Gly Gly Gly Thr Lys
  225                 230                 235

CTG GAG ATC TAGCTGATCA AAGCTCTAGA                                   748
Leu Glu Ile
      240
```

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 241 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: protein

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:11:

```
Ser Gln Val Gln Leu Gln Gln Ser Gly Ala Glu Leu Val Arg Pro Gly
  1                 5                  10                  15

Thr Ser Val Lys Leu Ser Cys Lys Ala Ser Asp Tyr Thr Phe Thr Ser
          20                  25                  30

Tyr Trp Met Asn Trp Val Lys Gln Arg Pro Gly Gln Gly Leu Glu Trp
      35                  40                  45

Ile Gly Met Ile Asp Pro Ser Asp Ser Glu Thr Gln Tyr Asn Gln Met
      50                  55                  60

Phe Lys Asp Lys Ala Ala Leu Thr Val Asp Lys Ser Ser Asn Thr Ala
  65                  70                  75                  80

Tyr Met Gln Leu Ser Ser Leu Thr Ser Glu Asp Ser Ala Val Tyr Tyr
          85                  90                  95

Cys Ala Lys Gly Gly Ala Ser Gly Asp Trp Tyr Phe Asp Val Trp Gly
      100                 105                 110

Gln Gly Thr Thr Val Thr Val Ser Ser Gly Gly Gly Gly Ser Gly Gly
  115                 120                 125

Gly Gly Ser Gly Gly Gly Gly Ser Asp Ile Gln Leu Thr Gln Ser Pro
      130                 135                 140

Ser Ser Leu Ser Ala Ser Leu Gly Gly Glu Val Thr Ile Thr Cys Lys
145                 150                 155                 160

Ala Ser Gln Asp Ile Lys Lys Tyr Ile Ala Trp Tyr Gln His Lys Pro
      165                 170                 175

Gly Lys Ser Pro Arg Leu Leu Ile His Tyr Thr Ser Val Leu Gln Pro
      180                 185                 190

Gly Ile Pro Ser Arg Phe Ser Gly Ser Gly Ser Gly Arg Asp Tyr Ser
```

-continued

```
   195                 200                 205

Phe Ser Ile His Asn Leu Glu Pro Glu Asp Ile Ala Thr Tyr Tyr Cys
     210                 215                 220

Leu His Tyr Asp Tyr Leu Tyr Thr Phe Gly Gly Gly Thr Lys Leu Glu
225                 230                 235                 240

Ile
```

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 201 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: DNA

(i i i) HYPOTHETICAL: NO

(i v) ANTI-SENSE: NO

(v i) ORIGINAL SOURCE:
(A) ORGANISM: Pseudomonas aeruginosa PAK
(C) INDIVIDUAL ISOLATE: E. coli

(v i i) IMMEDIATE SOURCE:
(B) CLONE: pWW22

(i x) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 1..201
(D) OTHER INFORMATION: /note="from 1 to 27 synthetic spacer; from 29 to 201 partial exotoxin A sequence corresponding to nucleotide positions 1574 to 1747 bp of the exotoxin A sequence (Gray et al.)

(x) PUBLICATION INFORMATION:
(A) AUTHORS: Gray, et al.
(C) JOURNAL: Proc. Natl. Acad. Sci. U.S.A.
(D) VOLUME: 81
(F) PAGES: 2645-2649
(G) DATE: 1984
(K) RELEVANT RESIDUES IN SEQ ID NO:12: FROM 29 TO 201

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:12:

```
AAGCTTAAGG AGATCTGCAT GCTTCTAGAG GGCGGCAGCC TGGCCGCGCT GACCGCGCAC     60

CAGGCCTGCC ACCTGCCGCT GGAGACTTTC ACCCGTCATC GCCAGCCGCG CGGCTGGGAA    120

CAACTGGAGC AGTGCGGCTA TCCGGTGCAG CGGCTGGTCG CCCTCTACCT GGCGGCGCGA    180

CTGTCATGGA ACCAGGTCGA C                                               201
```

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 2012 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: DNA

(i i i) HYPOTHETICAL: NO

(i v) ANTI-SENSE: NO

(v i) ORIGINAL SOURCE:
(A) ORGANISM: Mouse/Pseudomonas aeruginosa
(C) INDIVIDUAL ISOLATE: E. coli

(v i i) IMMEDIATE SOURCE:
(B) CLONE: pWW215-5

-continued

(i x) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 1..1911
(D) OTHER INFORMATION: /note="64-87 FLAG peptide and enterokinase cleavage site; 97 '53 heavy chain variable domain;454-498 15 aa linker sequence; 499-822 FRP5 light chain variable domain

(i x) FEATURE:
(A) NAME/KEY: sig_peptide
(B) LOCATION: 1..63
(D) OTHER INFORMATION: /note="ompA signal peptide"

(i x) FEATURE:
(A) NAME/KEY: mat_peptide
(B) LOCATION: 94..1911

(i x) FEATURE:
(A) NAME/KEY: 3'UTR
(B) LOCATION: 1912..2012
(D) OTHER INFORMATION: /function="3'non-coding region of the exotoxin A gene"

(i x) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 826..1911
(D) OTHER INFORMATION: /note="Exotoxin A gene coding region (coding for amino acids 252 to 613 of the mature exotoxin A)"

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:13:

```
ATG AAA AAG ACA GCT ATC GCG ATT GCA GTG GCA CTG GCT GGT TTC GCT        48
Met Lys Lys Thr Ala Ile Ala Ile Ala Val Ala Leu Ala Gly Phe Ala
-31 -30                 -25                 -20

ACC GTT GCG CAA GCT GAC TAC AAG GAC GAC GAT GAC AAG CTA GCT TCT        96
Thr Val Ala Gln Ala Asp Tyr Lys Asp Asp Asp Asp Lys Leu Ala Ser
-15                 -10                  -5                   1

CAG GTA CAA CTG CAG CAG TCT GGA CCT GAA CTG AAG AAG CCT GGA GAG       144
Gln Val Gln Leu Gln Gln Ser Gly Pro Glu Leu Lys Lys Pro Gly Glu
          5                  10                  15

ACA GTC AAG ATC TCC TGC AAG GCC TCT GGG TAT CCT TTC ACA AAC TAT       192
Thr Val Lys Ile Ser Cys Lys Ala Ser Gly Tyr Pro Phe Thr Asn Tyr
     20                  25                  30

GGA ATG AAC TGG GTG AAG CAG GCT CCA GGA CAG GGT TTA AAG TGG ATG       240
Gly Met Asn Trp Val Lys Gln Ala Pro Gly Gln Gly Leu Lys Trp Met
          35                  40                  45

GGC TGG ATT AAC ACC TCC ACT GGA GAG TCA ACA TTT GCT GAT GAC TTC       288
Gly Trp Ile Asn Thr Ser Thr Gly Glu Ser Thr Phe Ala Asp Asp Phe
     50                  55                  60                  65

AAG GGA CGG TTT GAC TTC TCT TTG GAA ACC TCT GCC AAC ACT GCC TAT       336
Lys Gly Arg Phe Asp Phe Ser Leu Glu Thr Ser Ala Asn Thr Ala Tyr
          70                  75                  80

TTG CAG ATC AAC AAC CTC AAA AGT GAA GAC ATG GCT ACA TAT TTC TGT       384
Leu Gln Ile Asn Asn Leu Lys Ser Glu Asp Met Ala Thr Tyr Phe Cys
          85                  90                  95

GCA AGA TGG GAG GTT TAC CAC GGC TAC GTT CCT TAC TGG GGC CAA GGG       432
Ala Arg Trp Glu Val Tyr His Gly Tyr Val Pro Tyr Trp Gly Gln Gly
     100                 105                 110

ACC ACG GTC ACC GTT TCC TCT GGC GGT GGC GGT TCT GGT GGC GGT GGC       480
Thr Thr Val Thr Val Ser Ser Gly Gly Gly Gly Ser Gly Gly Gly Gly
          115                 120                 125

TCC GGC GGT GGC GGT TCT GAC ATC CAG CTG ACC CAG TCT CAC AAA TTC       528
Ser Gly Gly Gly Gly Ser Asp Ile Gln Leu Thr Gln Ser His Lys Phe
130                 135                 140                 145

CTG TCC ACT TCA GTA GGA GAC AGG GTC AGC ATC ACC TGC AAG GCC AGT       576
Leu Ser Thr Ser Val Gly Asp Arg Val Ser Ile Thr Cys Lys Ala Ser
          150                 155                 160
```

-continued

```
CAG GAT GTG TAT AAT GCT GTT GCC TGG TAT CAA CAG AAA CCA GGA CAA          624
Gln Asp Val Tyr Asn Ala Val Ala Trp Tyr Gln Gln Lys Pro Gly Gln
      165                 170                 175

TCT CCT AAA CTT CTG ATT TAC TCG GCA TCC TCC CGG TAC ACT GGA GTC          672
Ser Pro Lys Leu Leu Ile Tyr Ser Ala Ser Ser Arg Tyr Thr Gly Val
  180                 185                 190

CCT TCT CGC TTC ACT GGC AGT GGC TCT GGG CCG GAT TTC ACT TTC ACC          720
Pro Ser Arg Phe Thr Gly Ser Gly Ser Gly Pro Asp Phe Thr Phe Thr
      195                 200                 205

ATC AGC AGT GTG CAG GCT GAA GAC CTG GCA GTT TAT TTC TGT CAG CAA          768
Ile Ser Ser Val Gln Ala Glu Asp Leu Ala Val Tyr Phe Cys Gln Gln
210                 215                 220                 225

CAT TTT CGT ACT CCA TTC ACG TTC GGC TCG GGG ACA AAA TTG GAG ATC          816
His Phe Arg Thr Pro Phe Thr Phe Gly Ser Gly Thr Lys Leu Glu Ile
      230                 235                 240

AAA GCT CTA GAG GGC GGC AGC CTG GCC GCG CTG ACC GCG CAC CAG GCC          864
Lys Ala Leu Glu Gly Gly Ser Leu Ala Ala Leu Thr Ala His Gln Ala
      245                 250                 255

TGC CAC CTG CCG CTG GAG ACT TTC ACC CGT CAT CGC CAG CCG CGC GGC          912
Cys His Leu Pro Leu Glu Thr Phe Thr Arg His Arg Gln Pro Arg Gly
  260                 265                 270

TGG GAA CAA CTG GAG CAG TGC GGC TAT CCG GTG CAG CGG CTG GTC GCC          960
Trp Glu Gln Leu Glu Gln Cys Gly Tyr Pro Val Gln Arg Leu Val Ala
      275                 280                 285

CTC TAC CTG GCG GCG CGA CTG TCA TGG AAC CAG GTC GAC CAG GTG ATC         1008
Leu Tyr Leu Ala Ala Arg Leu Ser Trp Asn Gln Val Asp Gln Val Ile
290                 295                 300                 305

CGC AAC GCC CTG GCC AGC CCC GGC AGC GGC GGC GAC CTG GGC GAA GCG         1056
Arg Asn Ala Leu Ala Ser Pro Gly Ser Gly Gly Asp Leu Gly Glu Ala
      310                 315                 320

ATC CGC GAG CAG CCC GAG CAG GCC CGT CTG GCC CTG ACC CTG GCC GCC         1104
Ile Arg Glu Gln Pro Gln Gln Ala Arg Leu Ala Leu Thr Leu Ala Ala
      325                 330                 335

GCC GAG AGC GAG CGC TTC GTC CGG CAG GGC ACC GGC AAC GAC GAG GCC         1152
Ala Glu Ser Glu Arg Phe Val Arg Gln Gly Thr Gly Asn Asp Glu Ala
  340                 345                 350

GGC GCG GCC AAC GCC GAC GTG GTG AGC CTG ACC TGC CCG GTC GCC GCC         1200
Gly Ala Ala Asn Ala Asp Val Val Ser Leu Thr Cys Pro Val Ala Ala
      355                 360                 365

GGT GAA TGC GCG GGC CCG GCG GAC AGC GGC GAC GCC CTG CTG GAG CGC         1248
Gly Glu Cys Ala Gly Pro Ala Asp Ser Gly Asp Ala Leu Leu Glu Arg
370                 375                 380                 385

AAC TAT CCC ACT GGC GCG GAG TTC CTC GGC GAC GGC GGC GAC GTC AGC         1296
Asn Tyr Pro Thr Gly Ala Glu Phe Leu Gly Asp Gly Gly Asp Val Ser
      390                 395                 400

TTC AGC ACC CGC GGC ACG CAG AAC TGG ACG GTG GAG CGG CTG CTC CAG         1344
Phe Ser Thr Arg Gly Thr Gln Asn Trp Thr Val Glu Arg Leu Leu Gln
      405                 410                 415

GCG CAC CGC CAA CTG GAG GAG CGC GGC TAT GTG TTC GTC GGC TAC CAC         1392
Ala His Arg Gln Leu Glu Glu Arg Gly Tyr Val Phe Val Gly Tyr His
  420                 425                 430

GGC ACC TTC CTC GAA GCG GCG CAA AGC ATC GTC TTC GGC GGG GTG CGC         1440
Gly Thr Phe Leu Glu Ala Ala Gln Ser Ile Val Phe Gly Gly Val Arg
      435                 440                 445

GCG CGC AGC CAG GAC CTC GAC GCG ATC TGG CGC GGT TTC TAT ATC GCC         1488
Ala Arg Ser Gln Asp Leu Asp Ala Ile Trp Arg Gly Phe Tyr Ile Ala
450                 455                 460                 465

GGC GAT CCG GCG CTG GCC TAC GGC TAC GCC CAG GAC CAG GAA CCC GAC         1536
Gly Asp Pro Ala Leu Ala Tyr Gly Tyr Ala Gln Asp Gln Glu Pro Asp
      470                 475                 480
```

-continued

```
GCA CGC GGC CGG ATC CGC AAC GGT GCC CTG CTG CGG GTC TAT GTG CCG      1584
Ala Arg Gly Arg Ile Arg Asn Gly Ala Leu Leu Arg Val Tyr Val Pro
      485                 490                 495

CGC TCG AGC CTG CCG GGC TTC TAC CGC ACC AGC CTG ACC CTG GCC GCG      1632
Arg Ser Ser Leu Pro Gly Phe Tyr Arg Thr Ser Leu Thr Leu Ala Ala
  500                 505                 510

CCG GAG GCG GCG GGC GAG GTC GAA CGG CTG ATC GGC CAT CCG CTG CCG      1680
Pro Glu Ala Ala Gly Glu Val Glu Arg Leu Ile Gly His Pro Leu Pro
      515                 520                 525

CTG CGC CTG GAC GCC ATC ACC GGC CCC GAG GAG GAA GGC GGG CGC CTG      1728
Leu Arg Leu Asp Ala Ile Thr Gly Pro Glu Glu Glu Gly Gly Arg Leu
530                 535                 540                 545

GAG ACC ATT CTC GGC TGG CCG CTG GCC GAG CGC ACC GTG GTG ATT CCC      1776
Glu Thr Ile Leu Gly Trp Pro Leu Ala Glu Arg Thr Val Val Ile Pro
      550                 555                 560

TCG GCG ATC CCC ACC GAC CCG CGC AAC GTC GGC GGC GAC CTC GAC CCG      1824
Ser Ala Ile Pro Thr Asp Pro Arg Asn Val Gly Gly Asp Leu Asp Pro
      565                 570                 575

TCC AGC ATC CCC GAC AAG GAA CAG GCG ATC AGC GCC CTG CCG GAC TAC      1872
Ser Ser Ile Pro Asp Lys Glu Gln Ala Ile Ser Ala Leu Pro Asp Tyr
  580                 585                 590

GCC AGC CAG CCC GGC AAA CCG CCG CGC GAG GAC CTG AAG TAACTGCCGC       1921
Ala Ser Gln Pro Gly Lys Pro Pro Arg Glu Asp Leu Lys
      595                 600                 605

GACCGGCCGG CTCCCTTCGC AGGAGCCGGC CTTCTCGGGG CCTGGCCATA CATCAGGTTT    1981

TCCTGATGCC AGCCCAATCG AATATGAATT C                                   2012
```

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 637 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: protein

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:14:

```
Met Lys Lys Thr Ala Ile Ala Ile Ala Val Ala Leu Ala Gly Phe Ala
-31 -30                 -25                 -20

Thr Val Ala Gln Ala Asp Tyr Lys Asp Asp Asp Asp Lys Leu Ala Ser
-15                 -10                  -5                   1

Gln Val Gln Leu Gln Gln Ser Gly Pro Glu Leu Lys Lys Pro Gly Glu
          5                  10                  15

Thr Val Lys Ile Ser Cys Lys Ala Ser Gly Tyr Pro Phe Thr Asn Tyr
     20                  25                  30

Gly Met Asn Trp Val Lys Gln Ala Pro Gly Gln Gly Leu Lys Trp Met
          35                  40                  45

Gly Trp Ile Asn Thr Ser Thr Gly Glu Ser Thr Phe Ala Asp Asp Phe
  50                  55                  60                  65

Lys Gly Arg Phe Asp Phe Ser Leu Glu Thr Ser Ala Asn Thr Ala Tyr
          70                  75                  80

Leu Gln Ile Asn Asn Leu Lys Ser Glu Asp Met Ala Thr Tyr Phe Cys
          85                  90                  95

Ala Arg Trp Glu Val Tyr His Gly Tyr Val Pro Tyr Trp Gly Gln Gly
     100                 105                 110

Thr Thr Val Thr Val Ser Ser Gly Gly Gly Gly Ser Gly Gly Gly Gly
          115                 120                 125

Ser Gly Gly Gly Gly Ser Asp Ile Gln Leu Thr Gln Ser His Lys Phe
 130                 135                 140                 145
```

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```
Leu Ser Thr Ser Val Gly Asp Arg Val Ser Ile Thr Cys Lys Ala Ser
      150                 155                 160

Gln Asp Val Tyr Asn Ala Val Ala Trp Tyr Gln Gln Lys Pro Gly Gln
      165                 170                 175

Ser Pro Lys Leu Leu Ile Tyr Ser Ala Ser Ser Arg Tyr Thr Gly Val
  180                 185                 190

Pro Ser Arg Phe Thr Gly Ser Gly Ser Gly Pro Asp Phe Thr Phe Thr
      195                 200                 205

Ile Ser Ser Val Gln Ala Glu Asp Leu Ala Val Tyr Phe Cys Gln Gln
210                 215                 220                 225

His Phe Arg Thr Pro Phe Thr Phe Gly Ser Gly Thr Lys Leu Glu Ile
      230                 235                 240

Lys Ala Leu Glu Gly Gly Ser Leu Ala Ala Leu Thr Ala His Gln Ala
      245                 250                 255

Cys His Leu Pro Leu Glu Thr Phe Thr Arg His Arg Gln Pro Arg Gly
  260                 265                 270

Trp Glu Gln Leu Glu Gln Cys Gly Tyr Pro Val Gln Arg Leu Val Ala
      275                 280                 285

Leu Tyr Leu Ala Ala Arg Leu Ser Trp Asn Gln Val Asp Gln Val Ile
290                 295                 300                 305

Arg Asn Ala Leu Ala Ser Pro Gly Ser Gly Gly Asp Leu Gly Glu Ala
      310                 315                 320

Ile Arg Glu Gln Pro Glu Gln Ala Arg Leu Ala Leu Thr Leu Ala Ala
      325                 330                 335

Ala Glu Ser Glu Arg Phe Val Arg Gln Gly Thr Gly Asn Asp Glu Ala
  340                 345                 350

Gly Ala Ala Asn Ala Asp Val Val Ser Leu Thr Cys Pro Val Ala Ala
      355                 360                 365

Gly Glu Cys Ala Gly Pro Ala Asp Ser Gly Asp Ala Leu Leu Glu Arg
370                 375                 380                 385

Asn Tyr Pro Thr Gly Ala Glu Phe Leu Gly Asp Gly Gly Asp Val Ser
      390                 395                 400

Phe Ser Thr Arg Gly Thr Gln Asn Trp Thr Val Glu Arg Leu Leu Gln
      405                 410                 415

Ala His Arg Gln Leu Glu Glu Arg Gly Tyr Val Phe Val Gly Tyr His
  420                 425                 430

Gly Thr Phe Leu Glu Ala Ala Gln Ser Ile Val Phe Gly Gly Val Arg
      435                 440                 445

Ala Arg Ser Gln Asp Leu Asp Ala Ile Trp Arg Gly Phe Tyr Ile Ala
450                 455                 460                 465

Gly Asp Pro Ala Leu Ala Tyr Gly Tyr Ala Gln Asp Gln Glu Pro Asp
      470                 475                 480

Ala Arg Gly Arg Ile Arg Asn Gly Ala Leu Leu Arg Val Tyr Val Pro
      485                 490                 495

Arg Ser Ser Leu Pro Gly Phe Tyr Arg Thr Ser Leu Thr Leu Ala Ala
  500                 505                 510

Pro Glu Ala Ala Gly Glu Val Glu Arg Leu Ile Gly His Pro Leu Pro
      515                 520                 525

Leu Arg Leu Asp Ala Ile Thr Gly Pro Glu Glu Glu Gly Gly Arg Leu
530                 535                 540                 545

Glu Thr Ile Leu Gly Trp Pro Leu Ala Glu Arg Thr Val Val Ile Pro
      550                 555                 560

Ser Ala Ile Pro Thr Asp Pro Arg Asn Val Gly Gly Asp Leu Asp Pro
```

-continued

```
        565                 570                 575

Ser Ser Ile Pro Asp Lys Glu Gln Ala Ile Ser Ala Leu Pro Asp Tyr
    580                 585                 590

Ala Ser Gln Pro Gly Lys Pro Pro Arg Glu Asp Leu Lys
    595                 600                 605
```

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 2012 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: DNA

(i i i) HYPOTHETICAL: NO

(i v) ANTI-SENSE: NO

(v i) ORIGINAL SOURCE:
(A) ORGANISM: Mouse/Pseudomonas acruginosa
(C) INDIVIDUAL ISOLATE: E. coli

(v i i) IMMEDIATE SOURCE:
(B) CLONE: pWW215-51

(i x) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 1..1911
(D) OTHER INFORMATION: /note="64-87 FLAG peptide and enterokinase cleavage site;97-456 FWP51 heavy chain variable domain;457-501 15 aa linker sequence;502- 822 FWP51 light chain variable domain

(i x) FEATURE:
(A) NAME/KEY: sig_peptide
(B) LOCATION: 1..63
(D) OTHER INFORMATION: /note="ompA signal peptide"

(i x) FEATURE:
(A) NAME/KEY: mat_peptide
(B) LOCATION: 94..1911

(i x) FEATURE:
(A) NAME/KEY: 3'UTR
(B) LOCATION: 1912..2012
(D) OTHER INFORMATION: /function="3'non-coding region of the exotoxin A gene"

(i x) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 826..1911
(D) OTHER INFORMATION: /note="Exotoxin A gene coding region (coding for amino acids 252 to 613 of the mature exotoxin A)"

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:15:

```
ATG AAA AAG ACA GCT ATC GCG ATT GCA GTG GCA CTG GCT GGT TTC GCT          48
Met Lys Lys Thr Ala Ile Ala Ile Ala Val Ala Leu Ala Gly Phe Ala
-31 -30                 -25                 -20

ACC GTT GCG CAA GCT GAC TAC AAG GAC GAC GAT GAC AAG CTA GCT TCT          96
Thr Val Ala Gln Ala Asp Tyr Lys Asp Asp Asp Asp Lys Leu Ala Ser
-15                 -10                 -5                  1

CAG GTA CAA CTG CAG CAG TCT GGG GCT GAG CTG GTG AGG CCT GGG ACT         144
Gln Val Gln Leu Gln Gln Ser Gly Ala Glu Leu Val Arg Pro Gly Thr
        5                   10                  15

TCA GTG AAG CTG TCC TGC AAG GCT TCT GAT TAC ACC TTC ACC AGC TAC         192
Ser Val Lys Leu Ser Cys Lys Ala Ser Asp Tyr Thr Phe Thr Ser Tyr
    20                  25                  30

TGG ATG AAC TGG GTG AAG CAG AGG CCT GGA CAA GGC CTT GAA TGG ATT         240
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Trp Met Asn Trp Val Lys Gln Arg Pro Gly Gln Gly Leu Glu Trp Ile
      35                  40                  45

GGT ATG ATT GAT CCT TCA GAC AGT GAA ACT CAA TAC AAT CAA ATG TTC      288
Gly Met Ile Asp Pro Ser Asp Ser Glu Thr Gln Tyr Asn Gln Met Phe
 50                  55                  60                  65

AAG GAC AAG GCC GCA TTG ACT GTA GAC AAG TCC TCC AAT ACA GCC TAC      336
Lys Asp Lys Ala Ala Leu Thr Val Asp Lys Ser Ser Asn Thr Ala Tyr
          70                  75                  80

ATG CAA CTC AGC AGC CTG ACA TCT GAG GAC TCT GCG GTC TAT TAC TGT      384
Met Gln Leu Ser Ser Leu Thr Ser Glu Asp Ser Ala Val Tyr Tyr Cys
          85                  90                  95

GCA AAA GGG GGG GCC TCT GGG GAC TGG TAC TTC GAT GTC TGG GGC CAA      432
Ala Lys Gly Gly Ala Ser Gly Asp Trp Tyr Phe Asp Val Trp Gly Gln
     100                 105                 110

GGG ACC ACG GTC ACC GTT TCC TCT GGC GGT GGC GGT TCT GGT GGC GGT      480
Gly Thr Thr Val Thr Val Ser Ser Gly Gly Gly Gly Ser Gly Gly Gly
     115                 120                 125

GGC TCC GGC GGT GGC GGT TCT GAC ATC CAG CTG ACC CAG TCT CCA TCC      528
Gly Ser Gly Gly Gly Gly Ser Asp Ile Gln Leu Thr Gln Ser Pro Ser
130                 135                 140                 145

TCA CTG TCT GCA TCT CTG GGA GGC GAA GTC ACC ATC ACT TGC AAG GCA      576
Ser Leu Ser Ala Ser Leu Gly Gly Glu Val Thr Ile Thr Cys Lys Ala
          150                 155                 160

AGC CAA GAC ATT AAG AAG TAT ATA GCT TGG TAC CAA CAC AAG CCT GGA      624
Ser Gln Asp Ile Lys Lys Tyr Ile Ala Trp Tyr Gln His Lys Pro Gly
          165                 170                 175

AAA AGT CCT CGG CTA CTC ATA CAC TAC ACA TCT GTA TTA CAG CCA GGC      672
Lys Ser Pro Arg Leu Leu Ile His Tyr Thr Ser Val Leu Gln Pro Gly
     180                 185                 190

ATC CCA TCC AGG TTC AGT GGA AGT GGG TCT GGG AGA GAT TAT TCC TTC      720
Ile Pro Ser Arg Phe Ser Gly Ser Gly Ser Gly Arg Asp Tyr Ser Phe
     195                 200                 205

AGC ATC CAC AAC CTG GAG CCT GAA GAT ATT GCA ACT TAT TAT TGT CTA      768
Ser Ile His Asn Leu Glu Pro Glu Asp Ile Ala Thr Tyr Tyr Cys Leu
210                 215                 220                 225

CAT TAT GAT TAT CTG TAC ACG TTC GGA GGG GGC ACC AAG CTG GAG ATC      816
His Tyr Asp Tyr Leu Tyr Thr Phe Gly Gly Gly Thr Lys Leu Glu Ile
          230                 235                 240

AAA GCT CTA GAG GGC GGC AGC CTG GCC GCG CTG ACC GCG CAC CAG GCC      864
Lys Ala Leu Glu Gly Gly Ser Leu Ala Ala Leu Thr Ala His Gln Ala
          245                 250                 255

TGC CAC CTG CCG CTG GAG ACT TTC ACC CGT CAT CGC CAG CCG CGC GGC      912
Cys His Leu Pro Leu Glu Thr Phe Thr Arg His Arg Gln Pro Arg Gly
     260                 265                 270

TGG GAA CAA CTG GAG CAG TGC GGC TAT CCG GTG CAG CGG CTG GTC GCC      960
Trp Glu Gln Leu Glu Gln Cys Gly Tyr Pro Val Gln Arg Leu Val Ala
     275                 280                 285

CTC TAC CTG GCG GCG CGA CTG TCA TGG AAC CAG GTC GAC CAG GTG ATC     1008
Leu Tyr Leu Ala Ala Arg Leu Ser Trp Asn Gln Val Asp Gln Val Ile
290                 295                 300                 305

CGC AAC GCC CTG GCC AGC CCC GGC AGC GGC GGC GAC CTG GGC GAA GCG     1056
Arg Asn Ala Leu Ala Ser Pro Gly Ser Gly Gly Asp Leu Gly Glu Ala
          310                 315                 320

ATC CGC GAG CAG CCG GAG CAG GCC CGT CTG GCC CTG ACC CTG GCC GCC     1104
Ile Arg Glu Gln Pro Glu Gln Ala Arg Leu Ala Leu Thr Leu Ala Ala
          325                 330                 335

GCC GAG AGC GAG CGC TTC GTC CGG CAG GGC ACC GGC AAC GAC GAG GCC     1152
Ala Glu Ser Glu Arg Phe Val Arg Gln Gly Thr Gly Asn Asp Glu Ala
     340                 345                 350

GGC GCG GCC AAC GCC GAC GTG GTG AGC CTG ACC TGC CCG GTC GCC GCC     1200
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Gly Ala Ala Asn Ala Asp Val Val Ser Leu Thr Cys Pro Val Ala Ala
     355                 360                 365

GGT GAA TGC GCG GGC CCG GCG GAC AGC GGC GAC GCC CTG CTG GAG CGC      1248
Gly Glu Cys Ala Gly Pro Ala Asp Ser Gly Asp Ala Leu Leu Glu Arg
370                 375                 380                 385

AAC TAT CCC ACT GGC GCG GAG TTC CTC GGC GAC GGC GGC GAC GTC AGC      1296
Asn Tyr Pro Thr Gly Ala Glu Phe Leu Gly Asp Gly Gly Asp Val Ser
     390                 395                 400

TTC AGC ACC CGC GGC ACG CAG AAC TGG ACG GTG GAG CGG CTG CTC CAG      1344
Phe Ser Thr Arg Gly Thr Gln Asn Trp Thr Val Glu Arg Leu Leu Gln
     405                 410                 415

GCG CAC CGC CAA CTG GAG GAG CGC GGC TAT GTG TTC GTC GGC TAC CAC      1392
Ala His Arg Gln Leu Glu Glu Arg Gly Tyr Val Phe Val Gly Tyr His
  420                 425                 430

GGC ACC TTC CTC GAA GCG GCG CAA AGC ATC GTC TTC GGC GGG GTG CGC      1440
Gly Thr Phe Leu Glu Ala Ala Gln Ser Ile Val Phe Gly Gly Val Arg
     435                 440                 445

GCG CGC AGC CAG GAC CTC GAC GCG ATC TGG CGC GGT TTC TAT ATC GCC      1488
Ala Arg Ser Glu Asp Leu Asp Ala Ile Trp Arg Gly Phe Tyr Ile Ala
450                 455                 460                 465

GGC GAT CCG GCG CTG GCC TAC GGC TAC GCC CAG GAC CAG GAA CCC GAC      1536
Gly Asp Pro Ala Leu Ala Tyr Gly Tyr Ala Gln Asp Gln Glu Pro Asp
     470                 475                 480

GCA CGC GGC CGG ATC CGC AAC GGT GCC CTG CTG CGG GTC TAT GTG CCG      1584
Ala Arg Gly Arg Ile Arg Asn Gly Ala Leu Leu Arg Val Tyr Val Pro
     485                 490                 495

CGC TCG AGC CTG CCG GGC TTC TAC CGC ACC AGC CTG ACC CTG GCC GCG      1632
Arg Ser Ser Leu Pro Gly Phe Tyr Arg Thr Ser Leu Thr Leu Ala Ala
  500                 505                 510

CCG GAG GCG GCG GGC GAG GTC GAA CGG CTG ATC GGC CAT CCG CTG CCG      1680
Pro Glu Ala Ala Gly Glu Val Glu Arg Leu Ile Gly His Pro Leu Pro
     515                 520                 525

CTG CGC CTG GAC GCC ATC ACC GGC CCC GAG GAG GAA GGC GGG CGC CTG      1728
Leu Arg Leu Asp Ala Ile Thr Gly Pro Glu Glu Glu Gly Gly Arg Leu
530                 535                 540                 545

GAG ACC ATT CTC GGC TGG CCG CTG GCC GAG CGC ACC GTG GTG ATT CCC      1776
Glu Thr Ile Leu Gly Trp Pro Leu Ala Glu Arg Thr Val Val Ile Pro
     550                 555                 560

TCG GCG ATC CCC ACC GAC CCG CGC AAC GTC GGC GGC GAC CTC GAC CCG      1824
Ser Ala Ile Pro Thr Asp Pro Arg Asn Val Gly Gly Asp Leu Asp Pro
     565                 570                 575

TCC AGC ATC CCC GAC AAG GAA CAG GCG ATC AGC GCC CTG CCG GAC TAC      1872
Ser Ser Ile Pro Asp Lys Glu Gln Ala Ile Ser Ala Leu Pro Asp Tyr
  580                 585                 590

GCC AGC CAG CCC GGC AAA CCG CCG CGC GAG GAC CTG AAG TAACTGCCGC       1921
Ala Ser Gln Pro Gly Lys Pro Pro Arg Glu Asp Leu Lys
     595                 600                 605

GACCGGCCGG CTCCCTTCGC AGGAGCCGGC CTTCTCGGGG CCTGGCCATA CATCAGGTTT    1981

TCCTGATGCC AGCCCAATCG AATATGAATT C                                   2012
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(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 637 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: protein

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:16:

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Met Lys Lys Thr Ala Ile Ala Ile Ala Val Ala Leu Ala Gly Phe Ala
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-31  -30                 -25                 -20

Thr Val Ala Gln Ala Asp Tyr Lys Asp Asp Asp Asp Lys Leu Ala Ser
-15                 -10                  -5                   1

Glu Val Gln Leu Gln Gln Ser Gly Ala Glu Leu Val Arg Pro Gly Thr
          5                   10                  15

Ser Val Lys Leu Ser Cys Lys Ala Ser Asp Tyr Thr Phe Thr Ser Tyr
     20                  25                  30

Trp Met Asn Trp Val Lys Gln Arg Pro Gly Gln Gly Leu Glu Trp Ile
     35                  40                  45

Gly Met Ile Asp Pro Ser Asp Ser Glu Thr Gln Tyr Asn Gln Met Phe
 50                  55                  60                  65

Lys Asp Lys Ala Ala Leu Thr Val Asp Lys Ser Ser Asn Thr Ala Tyr
          70                  75                  80

Met Gln Leu Ser Ser Leu Thr Ser Glu Asp Ser Ala Val Tyr Tyr Cys
          85                  90                  95

Ala Lys Gly Gly Ala Ser Gly Asp Trp Tyr Phe Asp Val Trp Gly Gln
     100                 105                 110

Gly Thr Thr Val Thr Val Ser Ser Gly Gly Gly Gly Ser Gly Gly Gly
     115                 120                 125

Gly Ser Gly Gly Gly Gly Ser Asp Ile Gln Leu Thr Gln Ser Pro Ser
130                 135                 140                 145

Ser Leu Ser Ala Ser Leu Gly Gly Glu Val Thr Ile Thr Cys Lys Ala
          150                 155                 160

Ser Gln Asp Ile Lys Lys Tyr Ile Ala Trp Tyr Gln His Lys Pro Gly
          165                 170                 175

Lys Ser Pro Arg Leu Leu Ile His Tyr Thr Ser Val Leu Gln Pro Gly
     180                 185                 190

Ile Pro Ser Arg Phe Ser Gly Ser Gly Ser Gly Arg Asp Tyr Ser Phe
     195                 200                 205

Ser Ile His Asn Leu Glu Pro Glu Asp Ile Ala Thr Tyr Tyr Cys Leu
210                 215                 220                 225

His Tyr Asp Tyr Leu Tyr Thr Phe Gly Gly Gly Thr Lys Leu Glu Ile
          230                 235                 240

Lys Ala Leu Glu Gly Gly Ser Leu Ala Ala Leu Thr Ala His Gln Ala
          245                 250                 255

Cys His Leu Pro Leu Glu Thr Phe Thr Arg His Arg Gln Pro Arg Gly
     260                 265                 270

Trp Glu Gln Leu Glu Glu Cys Gly Tyr Pro Val Gln Arg Leu Val Ala
     275                 280                 285

Leu Tyr Leu Ala Ala Arg Leu Ser Trp Asn Gln Val Asp Glu Val Ile
290                 295                 300                 305

Arg Asn Ala Leu Ala Ser Pro Gly Ser Gly Gly Asp Leu Gly Glu Ala
          310                 315                 320

Ile Arg Glu Gln Pro Glu Gln Ala Arg Leu Ala Leu Thr Leu Ala Ala
          325                 330                 335

Ala Glu Ser Glu Arg Phe Val Arg Gln Gly Thr Gly Asn Asp Glu Ala
     340                 345                 350

Gly Ala Ala Asn Ala Asp Val Val Ser Leu Thr Cys Pro Val Ala Ala
     355                 360                 365

Gly Glu Cys Ala Gly Pro Ala Asp Ser Gly Asp Ala Leu Leu Glu Arg
370                 375                 380                 385

Asn Tyr Pro Thr Gly Ala Glu Phe Leu Gly Asp Gly Gly Asp Val Ser
          390                 395                 400
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Phe Ser Thr Arg Gly Thr Gln Asn Trp Thr Val Glu Arg Leu Leu Gln
        405                 410                 415

Ala His Arg Gln Leu Glu Glu Arg Gly Tyr Val Phe Val Gly Tyr His
    420                 425                 430

Gly Thr Phe Leu Glu Ala Ala Gln Ser Ile Val Phe Gly Gly Val Arg
        435                 440                 445

Ala Arg Ser Gln Asp Leu Asp Ala Ile Trp Arg Gly Phe Tyr Ile Ala
450                 455                 460                 465

Gly Asp Pro Ala Leu Ala Tyr Gly Tyr Ala Gln Asp Gln Glu Pro Asp
        470                 475                 480

Ala Arg Gly Arg Ile Arg Asn Gly Ala Leu Leu Arg Val Tyr Val Pro
        485                 490                 495

Arg Ser Ser Leu Pro Gly Phe Tyr Arg Thr Ser Leu Thr Leu Ala Ala
    500                 505                 510

Pro Glu Ala Ala Gly Glu Val Glu Arg Leu Ile Gly His Pro Leu Pro
        515                 520                 525

Leu Arg Leu Asp Ala Ile Thr Gly Pro Glu Glu Glu Gly Gly Arg Leu
530                 535                 540                 545

Glu Thr Ile Leu Gly Trp Pro Leu Ala Glu Arg Thr Val Val Ile Pro
        550                 555                 560

Ser Ala Ile Pro Thr Asp Pro Arg Asn Val Gly Gly Asp Leu Asp Pro
        565                 570                 575

Ser Ser Ile Pro Asp Lys Glu Gln Ala Ile Ser Ala Leu Pro Asp Tyr
    580                 585                 590

Ala Ser Gln Pro Gly Lys Pro Pro Arg Glu Asp Leu Lys
        595                 600                 605
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We claim:

1. A recombinant antibody directed to the extracellular domain of the growth factor receptor c-erbB-2 comprising of heavy chain variable domain and a light chain variable domain of a monoclonal antibody, wherein the heavy chain variable domain comprises a polypeptide of the formula

$$FR_1\text{-}CDR_{1H}\text{-}FR_2\text{-}CDR_{2H}\text{-}FR_3\text{-}CDR_{3H}\text{-}FR_4 \quad (I)$$

wherein FR_1 is a polypeptide comprising 25–33 naturally occurring amino acids, FR_2 is a polypeptide comprising 12–16 naturally occurring amino acids, FR_3 is a polypeptide comprising 30–34 naturally occurring amino acids, FR_4 is a polypeptide comprising 6–13 naturally occurring amino acids, CDR_{1H} is a polypeptide of the amino acid sequence 32–36 of SEQ ID NO:5, CDR_{2H} is a polypeptide of the amino acid sequence 51–67 of SEQ ID NO:5, CDR_{3H} is a polypeptide of the amino acid sequence 100 to 109 of SEQ ID NO:5, and wherein the amino acid Cys may be in the oxidized state forming S—S-bridges.

2. A recombinant antibody according to claim 1 wherein the heavy chain variable domain comprises a polypeptide of the amino acid sequence 2 to 120 of SEQ ID NO:5, wherein optionally one or more single amino acids within the amino acid sequences 2 to 31 (FR_1), 37 to 50 (FR_2), 68 to 99 (FR_3), and/or 110 to 120 (FR_4) are replaced by other amino acids or deleted, and wherein the amino acid Cys may be in the oxidized state forming S—S-bridges.

3. A recombinant antibody according to claim 1 wherein the heavy chain variable domain comprises a polypeptide of the amino acid sequence 2 to 120 of SEQ ID NO:5, wherein the amino acid Cys may be in the oxidized state forming S—S-bridges.

4. A recombinant antibody directed to the extracellular domain of the growth factor receptor c-erbB-2 comprising of heavy chain variable domain and a light chain variable domain of a monoclonal antibody, wherein the light chain variable domain comprises a polypeptide of the formula

$$FR_6\text{-}CDR_{1L}\text{-}FR_7\text{-}CDR_{2L}\text{-}FR_8\text{-}CDR_{3L}\text{-}FR_9 \quad (II)$$

wherein FR_6 is a polypeptide comprising naturally occurring amino acids, FR_7 is a polypeptide comprising 13–17 naturally occurring amino acids, FR_8 is a polypeptide comprising 30–34 naturally occurring amino acids, FR_9 is a polypeptide comprising naturally occurring amino acids, CDR_{1L} is a polypeptide of the amino acid sequence 159 to 169 of SEQ ID NO:5, CDR_{2L} is a polypeptide of the amino acid sequence 185 to 191 of SEQ ID NO:5, and CDR_{3L} is a polypeptide of the amino acid sequence 224 to 232 of SEQ ID NO:5, and wherein the amino acid Cys may be in the oxidized state forming S—S-bridges.

5. A recombinant antibody according to claim 4 wherein the light chain variable domain comprises a polypeptide of the amino acid sequence 136 to 241 of SEQ ID NO:5, wherein optionally one or more single amino acids within the amino acid sequences 136 to 158 (FR_6), 170 to 184 (FR_7), 192 to 223 (FR_8), and/or 233 to 241 (FR_9) are replaced by other amino acids or deleted, and wherein the amino acid Cys may be in the oxidized state forming S—S-bridges.

6. A recombinant antibody according to claim 4 wherein the light chain variable domain comprises a polypeptide of the amino acid sequence 136 to 241 of SEQ ID NO:5, wherein the amino acid Cys may be in the oxidized state forming S—S-bridges.

7. A recombinant antibody directed to the extracellular domain of the growth factor receptor c-erbB-2 comprising of heavy chain variable domain and a light chain variable domain of a monoclonal antibody, wherein the heavy chain variable domain comprises a polypeptide of the formula

$$FR_1\text{-}CDR_{1H}\text{-}FR_2\text{-}CDR_{2H}\text{-}FR_3\text{-}CDR_{3H}\text{-}FR_4 \quad (I)$$

wherein FR_1 is a polypeptide comprising 25–33 naturally occurring amino acids, FR_2 is a polypeptide comprising 12–16 naturally occurring amino acids, FR_3 is a polypeptide comprising 30–34 naturally occurring amino acids, FR_4 is a polypeptide comprising 6–13 naturally occurring amino acids, CDR_{1H} is a polypeptide of the amino acid sequence 32 to 36 of SEQ ID NO:11, CDR_{2H} is a polypeptide of amino acid sequence 51 to 67 of SEQ ID NO:11, and CDR_{3H} is a polypeptide of the amino acid sequence 100 to 110 of SEQ ID NO:11, and wherein the amino acid Cys may be in the oxidized state forming S—S-bridges.

8. A recombinant antibody according to claim 7 wherein the heavy chain variable domain comprises a polypeptide of the amino acid sequence 2 to 121 of SEQ ID NO:11, wherein optionally one or more single amino acids within the amino acid sequences 2 to 31 (FR_1), 37 to 50 (FR_2), 68 to 99 (FR_3), and/or 111 to 121 (FR_4) are replaced by other amino acids or deleted, and wherein the amino acid Cys may be in the oxidized state forming S—S-bridges.

9. A recombinant antibody according to claim 7 wherein the heavy chain variable domain comprises a polypeptide of the amino acid sequence 2 to 121 of SEQ ID NO:11, wherein the amino acid Cys may be in the oxidized state forming S—S-bridges.

10. A recombinant antibody directed to the extracellular domain of the growth factor receptor c-erbB-2 comprising of heavy chain variable domain and a light chain variable domain of a monoclonal antibody, wherein the light chain variable domain comprises a polypeptide of the formula

$$FR_6\text{-}CD_{1L}\text{-}FR_7\text{-}CDR_{2L}\text{-}FR_8\text{-}CDR_{3L}\text{-}FR_9 \quad (II)$$

wherein FR_6 is a polypeptide comprising naturally occurring amino acids, FR_7 is a polypeptide comprising 13–17 naturally occurring amino acids, FR_8 is a polypeptide comprising 30–34 naturally occurring amino acids, FR_9 is a polypeptide comprising naturally occurring amino acids, CDR_{1L} is a polypeptide of the amino acid sequence 160 to 170 of SEQ ID NO:11, CDR_{2L} is a polypeptide of the amino acid sequence 186 to 192 of SEQ ID NO:11, and CDR_{3L} is a polypeptide of the amino sequence 225 to 232 of SEQ ID NO:11, and wherein the amino acid Cys may be in the oxidized state forming S—S-bridges.

11. A recombinant antibody according to claim 10 wherein the light chain variable domain comprises a polypeptide of the amino acid sequence 137 to 241 of SEQ ID NO:11, wherein optionally one or more single amino acids within the amino acid sequences 137 to 159 (FR_6), 171 to 185 (FR_7), 193 to 224 (FR_8), and/or 233 to 241 (FR_9) are replaced by other amino acids or deleted, and wherein the amino acid Cys may be in the oxidized state forming S—S-bridges.

12. A recombinant antibody according to claim 10 wherein the light chain variable domain comprises a polypeptide of the amino acid sequence 137 to 241 of SEQ ID NO:11, wherein the amino acid Cys may be in the oxidized state forming S—S-bridges.

13. A single-chain recombinant antibody directed to the extracellular domain of the growth factor receptor c-erbB-2 comprising of heavy chain variable domain and a light chain variable domain of a monoclonal antibody, wherein the heavy chain variable domain and the light chain variable domain are linked by a polypeptide spacer group, and further wherein the heavy chain variable domain and the light chain variable domain are derived from the mouse monoclonal antibody FRP5.

14. A single-chain recombinant antibody according to claim 13 comprising the heavy chain variable domain of the mouse monoclonal antibody FRP5, the 15 amino acid polypeptide consisting of three repetitive subunits of Gly-Gly-Gly-Gly-Ser, the light chain variable domain of the mouse monoclonal antibody FRP5, and an enzyme or a toxin, or a biologically active variant thereof.

15. A single-chain recombinant antibody designated Fv(FRP5)-phoA according to claim 14 comprising a polypeptide of the amino acid sequence 2 to 690 of SEQ ID NO:7.

16. A single-chain recombinant antibody designated Fv(FRP5)-ETA according to claim 14 comprising a polypeptide of the amino acid sequence 2 to 606 of SEQ ID NO:14.

17. A single-chain recombinant antibody directed to the extracellular domain of the growth factor receptor c-erbB-2 comprising of heavy chain variable domain and a light chain variable domain of a monoclonal antibody, wherein the heavy chain variable domain and the light chain variable domain are linked by a polypeptide spacer group, and further wherein the heavy chain variable domain and the light chain variable domain are derived from the mouse monoclonal antibody FWP51.

18. A single-chain recombinant antibody according to claim 17 comprising the heavy chain variable domain of the mouse monoclonal antibody FWP51, the 15 amino acid polypeptide consisting of three repetitive subunits of Gly-Gly-Gly-Gly-Ser, the light chain variable domain of the mouse monoclonal antibody FWP51, and an enzyme or a toxin, or a biologically active variant thereof.

19. A single-chain recombinant antibody designated Fv(FWP51)-ETA according to claim 18 comprising a polypeptide of the amino acid sequence 2 to 606 of SEQ ID NO:16.

* * * * *

[CANCER RESEARCH 53, 334-339, January 15, 1993]

BR96 sFv-PE40, a Potent Single-Chain Immunotoxin That Selectively Kills Carcinoma Cells

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ABSTRACT

We have constructed a single-chain immunotoxin composed of the carcinoma-reactive antibody BR96 and a truncated form of *Pseudomonas* exotoxin. The chimeric molecule, BR96 sFv-PE40, was expressed in *Escherichia coli* and localized to the inclusion bodies. We purified and identified two species of BR96 sFv-PE40, monomers and aggregates. The monomeric form was able to bind well to the BR96 antigen, a Lewis^y-related antigen, while the aggregate was not. The binding affinity of the monomeric recombinant immunotoxin was 5-fold less than intact BR96 IgG, and its specificity for the BR96 antigen was confirmed by competition analysis. Monomeric BR96 sFv-PE40 was found to be extremely cytotoxic against cancer cells displaying the BR96 antigen. The cytotoxicity of the fusion protein correlates directly with antigen density on the tumor cell lines tested. The breast carcinoma cell line MCF-7, which has the highest density of BR96 antigen, was the most sensitive to BR96 sFv-PE40, with a concentration producing 50% protein synthesis inhibition of 5 pM. BR96 sFv-PE40 was found to have a $t_{1/2}$ in serum of 28.5 min in athymic mice, compared to that of the chemical conjugate, chiBR96-LysPE40, which was 54 min. These data indicate that the single-chain immunotoxin BR96 sFv-PE40 is a potent inhibitor of protein synthesis in target cell lines and may be an effective agent for the treatment of cancer.

INTRODUCTION

Immunotoxins are cytotoxic agents designed to kill specific populations of cells that display markers such as cell surface antigens or receptors (1, 2). These targeted molecules are composed of antibodies that have been chemically or biologically linked to a cytotoxic agent. One such agent, PE,[2] is produced by *Pseudomonas aeruginosa* and kills cells by ADP-ribosylating elongation factor 2, thereby inhibiting protein synthesis (3). Crystallographic analysis of PE revealed that the protein is composed of three domains (4). Domain I is responsible for cell binding, domain II for processing and translocation of the toxin, and domain III for inhibition of protein synthesis (5). The construction of immunotoxins often uses a binding-defective form of PE, known as PE40 (6). Therefore, when PE40 is attached to an antibody or antibody fragment, one can produce a fusion protein which both binds and is cytotoxic toward tumor cell lines bearing a specific antigen.

Most studies using immunotoxins to target tumor cell populations have been performed with molecules in the form of chemical conjugates (7–10). The antibody and toxin moieties of such reagents are typically joined by thioether or disulfide bonds. More recently, genetic engineering has been used to prepare single-chain immunotoxins that are constructed by fusing the two functional components, such as a single-chain antibody and a binding-defective toxin, at the DNA level and then producing the cytotoxic molecule as a chimeric protein (11–15). Single-chain immunotoxins offer the advantage of homogeneity because all the molecules produced are single chain proteins composed of the same amino acid residues. In a chemical conjugation reaction, it is difficult to generate a homogeneous population of molecules. In addition, since single-chain immunotoxins are approximately 3-fold smaller than chemical conjugates produced with intact IgG, they may be able to penetrate solid tumors more readily (16–19). Finally, single-chain immunotoxins have been demonstrated in some cases to be more potent reagents than equivalent chemical conjugates (11, 13, 14).

We have recently described the *in vitro* activities of an immunotoxin conjugate, chiBR96-LysPE40 (20), composed of a chimeric (mouse-human) form of BR96, made through homologous recombination (21), and LysPE40, a modified form of PE40 in which a lysine residue was engineered onto the amino terminus of the protein for conjugation purposes (22). BR96 IgG selectively binds to a tumor-associated antigen expressed on a high proportion of human lung, breast, and colon carcinoma cell lines (23). Administration of BR96 results in only modest antitumor activity against human tumor xenografts in mice (24). Because of the ability of the antibody to bind to carcinoma cells and rapidly internalize into the endocytic vesicles, BR96 is an excellent targeting reagent. In addition, when BR96 is chemically linked to drugs, the resulting molecule is a powerful antitumor agent *in vivo*.[3]

In this report, we describe the construction and characterization of BR96 sFv-PE40, a single-chain immunotoxin in which PE40 is fused to the cloned variable heavy and light chain antibody fragments (Fv) of the carcinoma-reactive antibody BR96. We show that BR96 sFv-PE40 specifically binds to its antigen and is cytotoxic against carcinoma cell lines that express this antigen on their surface. The bifunctional retention of both the specificity of the cell-binding portion of the molecule and the cytotoxic potential of the exotoxin makes for a very potent, targeted reagent.

MATERIALS AND METHODS

Reagents and Cell Lines. The immunotoxin conjugate chiBR96-LysPE40 was prepared exactly as described previously (20). Q-Sepharose was purchased from Pharmacia (Uppsala, Sweden). TSK-3000 columns were purchased from TosoHaas, Inc. (Philadelphia, PA). Immunoblots were performed using mouse anti-idiotypic BR96 antibody (757-4-1), which was kindly provided by B. Mixan, Bristol-Myers Squibb (Seattle, WA). ABC immunoblot kits were purchased from Vector Laboratories (Burlingame, CA). Chloramine T was purchased from Sigma Chemical Company (St. Louis, MO). MCF-7 human breast carcinoma cells were purchased from American Type Culture Collection (Rockville, MD). RCA colon carcinoma was obtained from M. Brattain (Baylor University). L2987 lung adenocarcinoma was obtained from I. Hellstrom, Bristol-Myers Squibb. A2780 ovarian carcinoma was obtained from K. Scanlon, National Institutes of Health (Bethesda, MD), and KB epidermoid carcinoma was obtained from I. Pastan, National Institutes of Health.

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[2] The abbreviations used are: PE, *Pseudomonas* exotoxin; PCR, polymerase chain reaction; PBS, phosphate-buffered saline; ELISA, enzyme-linked immunosorbent assay; BSA, bovine serum albumin; WTB buffer, PBS-0.05% Tween-20-1% BSA; FACS, fluorescence-activated cell sorter; ID, inhibition dose; ID_{50}, concentration producing 50% inhibition; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; V_H, variable heavy chain; V_L, variable light chain.

[3] P. Trail, D. Willner, S. J. Lasch, A. J. Henderson, A. M. Cassazza, R. A. Firestone, I. Hellström, and K. E. Hellström, manuscript in preparation.

Construction of the Plasmid Encoding BR96 sFv-PE40. The BR96 sFv sequence is encoded in the plasmid pBR96Fv.[4] A 550-base pair sequence encoding BR96 sFv was PCR amplified with primer 1 (5' GCTAGACATATG GAGGTGCAGCTGGT GGAGTCT-3') and primer 2 (5' GCTGTG-GAGACTGGCCTGGTTTCTGCAGG TACC-3'). The 5'-PCR primer (primer 1) was designed to encode a unique *Nde*I restriction site adjacent to an ATG translational initiation codon and the first seven codons of the variable heavy chain gene. The 3'-PCR primer (primer 2) was designed to anneal just downstream of the internal *Kpn*I site located within the variable light chain gene. After PCR amplification and digestion with *Nde*I and *Kpn*I, the 550-base pair *Nde*I *Kpn*I fragment was ligated into a 4220-base pair *Nde*I-*Kpn*I vector fragment prepared from plasmid pMS 8 (24), which encodes the gene for PE40 under the transcriptional control of the T7 promoter (25). The product of this ligation was an intermediate vector designated pBW 7.01. Subsequently, the 227-base pair *Kpn*I fragment from pBR96 Fv was subcloned into the unique *Kpn*I site of pBW 7.01. The resulting plasmid, pBW 7.0, encoding the BR96 sFv-PE40 gene fusion, was confirmed by DNA sequence analysis.

Expression and Purification of BR96 sFv-PE40. The expression plasmid pBW 7.0, encoding BR96 sFv-PE40, was transformed into *Escherichia coli* BL21 (ADE3) cells and cultured in Super Broth (Digene, Inc., Silver Spring, MD) containing 75 μg/ml of ampicillin at 37°C. When absorbance at 650 nm reached 1.0, isopropyl 1-thio-β-D-galactopyranoside was added to a final concentration of 1 mM, and cells were harvested 90 min later. The bacteria were washed in sucrose buffer (20% sucrose-30 mM Tris-HCl (pH 7.4)-1 mM EDTA) and osmotically shocked in ice-cold H_2O. Subsequently, inclusion bodies were isolated away from the spheroplast membrane proteins by extensive treatment with the nonionic detergent Tergitol (Sigma) to remove excess bacterial proteins, followed by denaturation in 7 M guanidine-HCl (pH 7.4), refolding in PBS supplemented with 0.4 M L-arginine, and extensive dialysis against 0.02 M Tris-HCl (pH 7.4). The protein was purified by anion-exchange (Q-Sepharose) and gel filtration (TSK-3000) chromatography with a Pharmacia fast protein liquid chromatography system, as described previously (26). Protein quantitation was determined using Bradford analysis (27).

Direct Lewis^y Antigen-binding ELISA. Lewis^y antigen obtained from ChemBiomed (Alberta, Canada), was diluted to 0.2 μg/ml in coating buffer (100 mM sodium bicarbonate, pH 9.4) prior to coating Dynatech Immulon II plates and incubating for 16 h at 4°C. Excess antigen was removed, and the plates were blocked with PTB buffer for 1 h at room temperature followed by 3 washes with PTB. The antibody samples were serially diluted in PTB to a final concentration ranging from 1.25 to 80 μg/ml and incubated overnight at 4°C on the plate in a volume of 50 μl/well. The plates were washed 3 times with PTB, each well incubated with 100 μl of biotinylated BR96 anti-idiotypic antibody (2.56 μg/ml) in PTB for 1 h at room temperature, and then washed 4 additional times with PTB. Alkaline phosphatase-conjugated streptavidin (Kirkegaard and Perry Laboratories, Gaithersburg, MD) was added to each well (100 μl of 0.5 μg/ml in PBS containing 1% BSA) and incubated for 1 h at 37°C. Plates were washed 3 times with PTB, 3 times with phosphatase buffer (75 mM Tris-0.1 M NaCl-5 mM $MgCl_2$, pH 9.4), and reacted with *p*-nitrophenyl phosphate (1 mM in phosphatase buffer) for 30–60 min at 37°C. The reaction was stopped by the addition of 2 N NaOH. The plates were read at 405 nm on a Molecular Devices, Inc. (Menlo Park, CA), microplate reader.

Binding Competition Analysis. Microtiter plates were coated with Lewis^y antigen as described above. Antibody samples were diluted in PBS containing 1% BSA to final concentrations ranging from 1.36 to 175 μg/ml. ^{125}I-BR96 IgG was added to each sample (5 μCi/ml) along with antibody competitor in a final volume of 100 μl. The entire mixture of radiolabeled BR96 IgG and antibody competitor was added to the Lewis^y-coated plates and incubated for 2 h at 37°C. The plates were washed 5 times with PBS containing 0.05% Tween-20, and the wells were counted on a gamma counter.

FACS Analysis. Assays were performed by fluorescence as previously described (23). Briefly, target cells were harvested in logarithmic phase with EDTA (0.02%) in calcium- and magnesium-free PBS. The cells were washed twice in PBS containing 1% BSA and resuspended to 1×10^7 cells/ml in PBS containing 1% BSA and 0.02% NaN_3. Cells (0.1 ml) were mixed with BR96 or a human IgG control (0.1 ml at 50 μg/ml) and incubated for 45 min at 4°C. The cells were washed 2 times and resuspended in 0.1 ml of an appropriate concentration of fluorescein isothiocyanate-labeled rabbit anti-human IgG (Cappel, Malvern, PA). Cells were incubated for 30 min at 4°C, washed 2 times in PBS containing 0.02% NaN_3, and analyzed on a Coulter EPICS 753 fluorescence-activated cell sorter. Data are expressed as the fluorescence intensity of cells reacted with BR96 minus cells reacted with control antibody. On a logarithmic scale, 25 units of fluorescence intensity represents a doubling of antigen density.

Inhibition of Protein Synthesis Assay. All cell lines were cultured as monolayers at 37°C in RPMI 1640 supplemented with 10% fetal bovine serum, 2 mM L-glutamine, and 50 units/ml penicillin/streptomycin. Tumor cells were plated onto 96-well flat-bottom tissue culture plates (1×10^4 cells/well) and kept at 37°C for 16 h. Dilutions of immunotoxin were made in growth media, and 0.1 ml was added to each well for 20 h at 37°C. Each dilution was done in triplicate. The cells were pulsed with [^{3}H]leucine (1 μCi/well) for an additional 4 h at 37°C. The cells were lysed by freeze-thawing and harvested using a Tomtec cell harvester (Orange, CT). Incorporation of [^{3}H]leucine was determined by a LKB Beta-Plate liquid scintillation counter.

For the competition experiment, tumor cells were prepared as described above. BR96 IgG or, as a control, L6 IgG was diluted to 100 μg/ml in growth media before addition to the cell monolayer (0.1 ml/well). After the material was incubated at 37°C for 1 h, dilutions of BR96 sFv-PE40 were added and incubated an additional hour, cell supernatants were removed, and cells were washed with complete RPMI growth media. Growth media (0.2 ml) was added to each well, and the cells were incubated at 37°C for 20 h and labeled with [^{3}H]leucine as described above.

^{125}I-Labeling of BR96 Immunotoxins. BR96 sFv-PE40 and chiBR96-LysPE40 were labeled with Na^{125}I using chloramine T (28). Each reaction contained 100 μg of immunotoxin in PBS, 1 μCi of Na^{125}I, and 10 ng/ml of chloramine T in a total reaction volume of 100 μl. After a 5-min incubation at room temperature, the reaction was terminated by addition of 20 ng/ml of Na-metabisulfite. The free Na^{125}I was separated from the radiolabeled immunotoxin by gel filtration through PD-10 columns (Pharmacia). The specific activity of both immunotoxins was approximately 10 μCi/μg.

Immunotoxin Blood Levels. Female athymic mice (*nu/nu*) were purchased from Harlan Sprague-Dawley (Indianapolis, IN) when they were 4–6 weeks of age. ^{125}I-BR96 sFv-PE40 or ^{125}I-chiBR96-LysPE40 (10 μCi) was injected i.v. via the tail vein. The animals (2–4/data point) were sacrificed at various time points, and the blood was collected and counted in a gamma counter. The % ID for the blood was determined as (cpm detected/cpm injected) × 100. We calculated % ID/ml assuming a 1.6-ml total blood volume.

RESULTS

Cloning and Expression of BR96 sFv-PE40 Chimeric Protein. The gene fusion encoding BR96 sFv-PE40 is composed of the BR96 variable region directly fused to the gene encoding PE40. We first isolated the sequences encoding the V_L and V_H domains of BR96 IgG from a plasmid containing the BR96 Fv sequences (pBR96Fv). The light and heavy chains were connected via a flexible polypeptide linker. The PCR-amplified fragments were subcloned into the vector pMS 8, which contains the gene encoding PE40. The resulting plasmid, pBW 7.0, and the construction scheme are depicted in Fig. 1.

Upon induction with isopropyl 1-thio-β-D-galactopyranoside, *E. coli* BL21 (ADE3) cells transformed with pBW 7.0 expressed fusion protein that was mainly localized to the inclusion bodies (data not shown). The protein was prepared, as described in "Materials and Methods," and subjected to ion-exchange chromatography on a Q-Sepharose column. Fractions containing BR96 sFv-PE40 were pooled and subsequently separated by gel filtration chromatography on a TSK-3000 column. The chromatographic profile of the size exclusion column shows the presence of two major species (Fig. 2*A*). The first species elutes between the M_r 660,000 and 158,000 gel filtration standards and represents an aggregated form of the recombinant protein (fractions 9–14). The second species (fractions 15–21) represents the M_r 67,000 monomeric form of BR96 sFv-PE40 which, as expected, eluted between the M_r 158,000 and 44,000 standards. These results were confirmed by reducing (Fig. 2*B*) and nonreducing SDS-PAGE analysis (Fig. 2*C*). Whereas Fig. 2*B* shows the purification profile as analyzed by SDS-PAGE and Coomassie staining, Fig. 2*C*

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shows an immunoblot analysis using anti-idiotypic BR96 antibody. Therefore, we have demonstrated that purification yields two forms of recombinant protein, monomers and aggregates. After pooling the monomer and aggregate peaks, we determined the homogeneity of the pools to be >95% by nonreducing SDS-PAGE (data not shown). The yield of correctly folded BR96 sFv-PE40 protein using these conditions is approximately 0.4% of the cell paste and 3% of the total inclusion body protein.

Binding Activity of BR96 sFv-PE40 toward Lewis^y Antigen. BR96 sFv-PE40, being monovalent, only provides one antigen-binding site per molecule. In order to test the binding avidity of this molecule, we performed a direct binding assay in which purified Lewis^y antigen was coated onto ELISA plates. In comparison with BR96 IgG, monomeric BR96 sFv-PE40 binds approximately 5-fold less well (Fig. 3*A*). In contrast, the aggregated form of BR96 sFv-PE40 was unable to bind to the Lewis^y antigen. L6 IgG, an antibody that does not bind the BR96 antigen, was used as a negative control.

Additionally, we compared the competitive binding ability of BR96 sFv-PE40 with BR96 IgG. This assay, in which Lewis^y-coated plates were also used, measured the amount of radioiodinated BR96 IgG that remained bound to the plate when competed with various amounts of BR96 sFv-PE40 or BR96 IgG. We found that BR96 sFv-PE40 competed 5-fold less well than BR96 IgG (Fig. 3*B*) which correlates with the direct binding data in Fig. 3*A*. The addition of L6 IgG, which did not compete for binding, demonstrates the specificity of this assay.

Cytotoxicity of BR96 sFv-PE40 against Cancer Cells. To determine the cytotoxic potential of monomeric BR96 sFv-PE40, we compared the effect of the immunotoxin to the chemical conjugate, chi-BR96-LysPE40 (20) on MCF-7 breast carcinoma cells (Fig. 4). The results indicate that the single-chain immunotoxin is 4-fold more potent than the conjugate, which was prepared in a 1:1 ratio of toxin to antibody, with ID_{50} values of 5 and 20 pM, respectively. Next, in order to correlate cytotoxicity with the presence of the BR96 antigen, we determined the relative antigen density on five tumor cell lines by FACS analysis (Fig. 5), using a fluorescein isothiocyanate-labeled rabbit anti-human IgG. FACS analysis of a nonspecific human IgG antibody was performed for each cell line to determine nonspecific fluorescence, and a fluorescence intensity was calculated (Table 1).

When we tested the cytotoxic potential of BR96 sFv-PE40 on these cell lines, we found that inhibition of protein synthesis correlated with BR96 antigen density (Table 1). For example, MCF-7 cells were found to display the highest density of BR96 antigen and were the most sensitive to BR96 sFv-PE40 (ID_{50} of 5 pM) of the cell lines tested. In contrast, KB cells which display negligible amounts of the BR96 antigen were much less sensitive to BR96 sFv-PE40 (ID_{50} of 7,462 pM). We also compared the cytotoxic activity of monomeric and aggregated BR96 sFv-PE40 and found that the monomer was approximately 40- to 50-fold more effective at inhibiting protein synthesis than the aggregate population with ID_{50} values on L2987 cells of 75 and 2920 pM, respectively (data not shown).

To confirm the specificity of the immunotoxin for its antigen-binding site, competitive cytotoxicity experiments were carried out (Fig. 6). We determined that the cytotoxic effect of BR96 sFv-PE40 was due to specific antigen binding, since the effect is reduced by excess BR96 IgG but not by L6 IgG, which does not recognize the BR96 antigen.

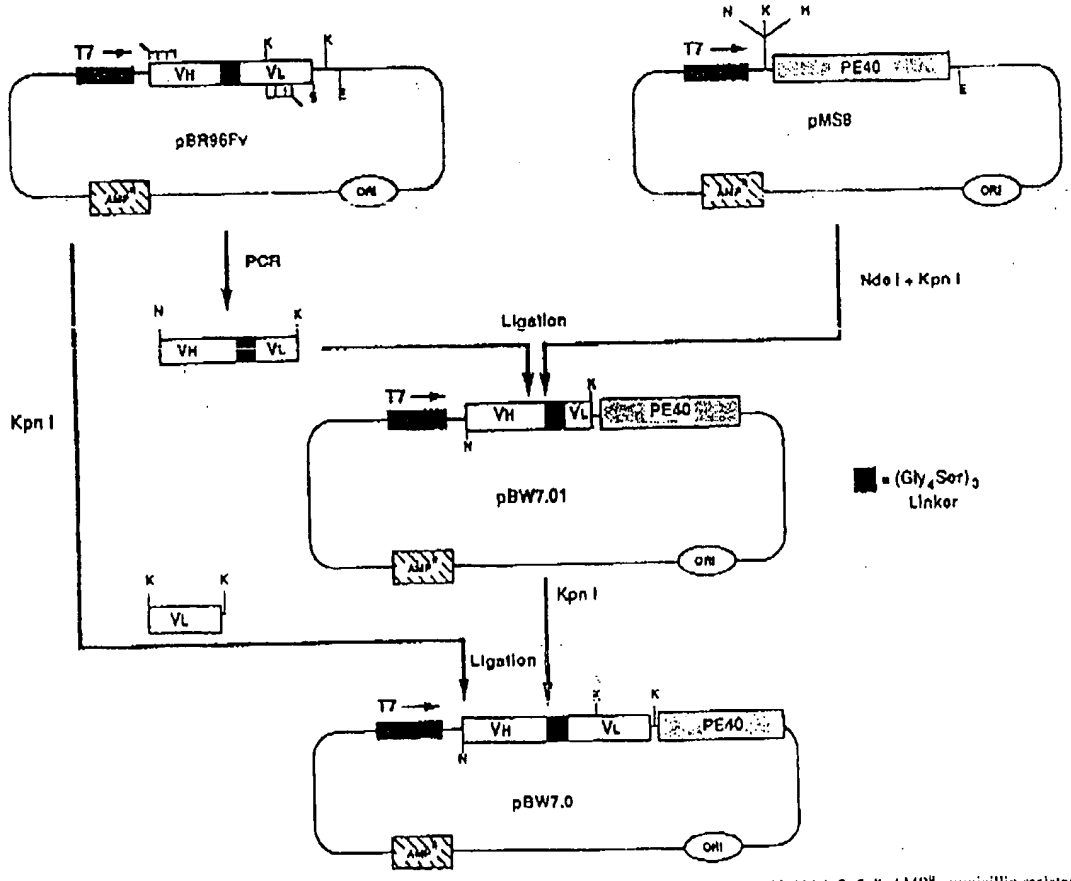

Fig. 1. Construction scheme of expression plasmid pBW 7.0 encoding BR96 sFv-PE40. E, *Eco*RI; H, *Hind*III; K, *Kpn*I; N, *Nde*I; S, *Sal*I; AMP^R, ampicillin resistent gene. $(Gly_4Ser)_3$ represents a 15-amino acid linker.

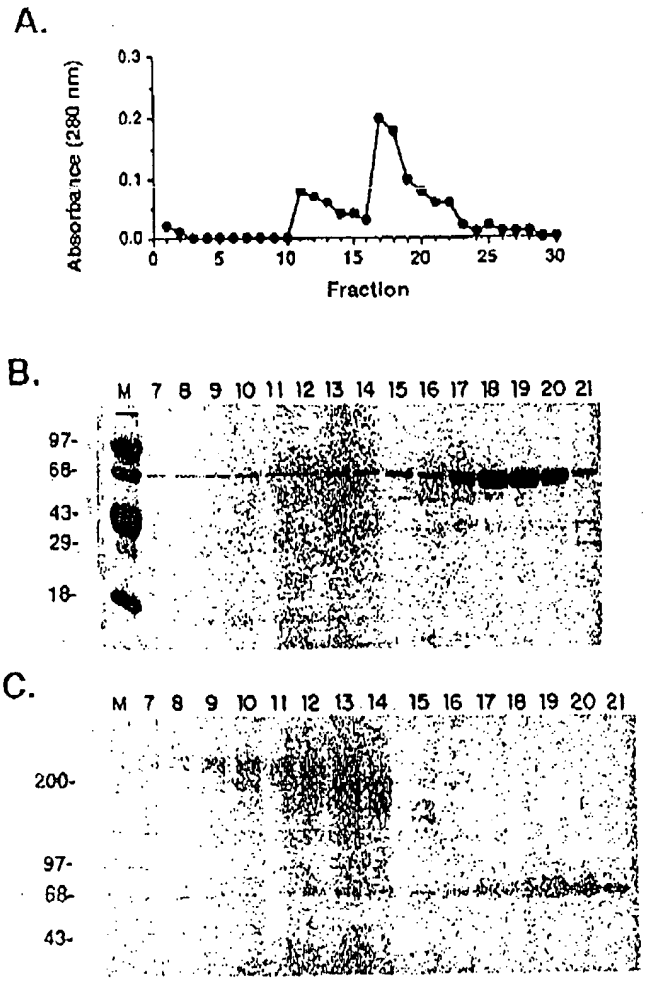

Fig. 2. Purification of BR96 sFv-PE40. *A*, gel filtration column chromatography of renatured BR96 sFv-PE40 after initial purification over Q-Sepharose. *B*, 12% reducing SDS-polyacrylamide gel stained with Coomassie brilliant blue. *C*, immunoblot of a 4–12% nonreducing SDS-polyacrylamide gel probed with BR96 anti-idiotypic antibody. The lanes correspond to fractions 7–21 on the gel filtration profile in *A*; *Lane M*, molecular weight marker proteins in thousands. Molecular weight standards corresponding to 670,000, 158,000, 44,000, and 17,000 eluted in fractions 10, 15, 21, and 30, respectively.

Comparative Blood Level Lifetime Analysis of BR96-Immunotoxins. BR96 sFv-PE40 is approximately one-third the size of the immunotoxin conjugate, chiBR96-LysPE40. Since protein size can effect biological kinetics, we measured the difference in blood half-life between BR96 sFv-PE40 and chiBR96-LysPE40. Both immunotoxins were radioiodinated and administered to athymic mice via their tail vein. Blood samples were collected at various times and counted (Table 2). We found that BR96 sFv-PE40 clears from the blood faster than chiBR96-LysPE40. We performed pharmacokinetic analysis on the data using the PKCALC statistical analysis program (29). The analysis showed that a two-compartment model was appropriate for the data. For chiBR96-LysPE40, the $t_{1/2}$ α and β were 54 min and 16.6 h, respectively. For BR96 sFv-PE40, the $t_{1/2}$ α and β were 28.5 min and 8.2 h, respectively.

In the above experiment, the measurement of ^{125}I-labeled BR96 immunotoxin in the blood determined how much of the molecule was present. In order to measure the amount of detectable single-chain immunotoxin that was biologically active, we assayed the blood for BR96 sFv-PE40-directed cytotoxic activity at the various times indicated. We found that all of the detectable immunotoxin retained its biological activity (data not shown).

DISCUSSION

We have constructed an expression plasmid for the production of a single-chain immunotoxin composed of the carcinoma-reactive antibody BR96 and a truncated form of *Pseudomonas* exotoxin. The chimeric molecule, BR96 sFv-PE40, was purified from *E. coli* and was found to exist in both a monomeric and aggregated form. The aggregate peak contained both disulfide bonded and noncovalent forms, as determined by nonreducing SDS-PAGE (Fig. 2C). The monomeric form was found to bind 5-fold less well than BR96 IgG, while the aggregated form did not bind to the BR96 antigen. The specificity of monomeric BR96 sFv-PE40 for its antigen was confirmed through a competition analysis with BR96 IgG.

Since BR96 IgG binds to its antigen in a bivalent fashion, we were uncertain as to whether the monovalent sFv molecule would be able to bind well. Other investigators (11, 14, 30, 31) have reported a reduction in binding efficiency of recombinant antibody proteins of 3- to 10-fold relative to their IgG counterparts. One might have expected the aggregates, since they are multivalent, to bind better than monovalent BR96 sFv-PE40. However, the BR96 sFv-PE40 aggregates mainly result from single molecules that were misfolded during production, forming inappropriate disulfide bonds. Since there are 8 cysteine residues in BR96 sFv-PE40, the aggregated material was not

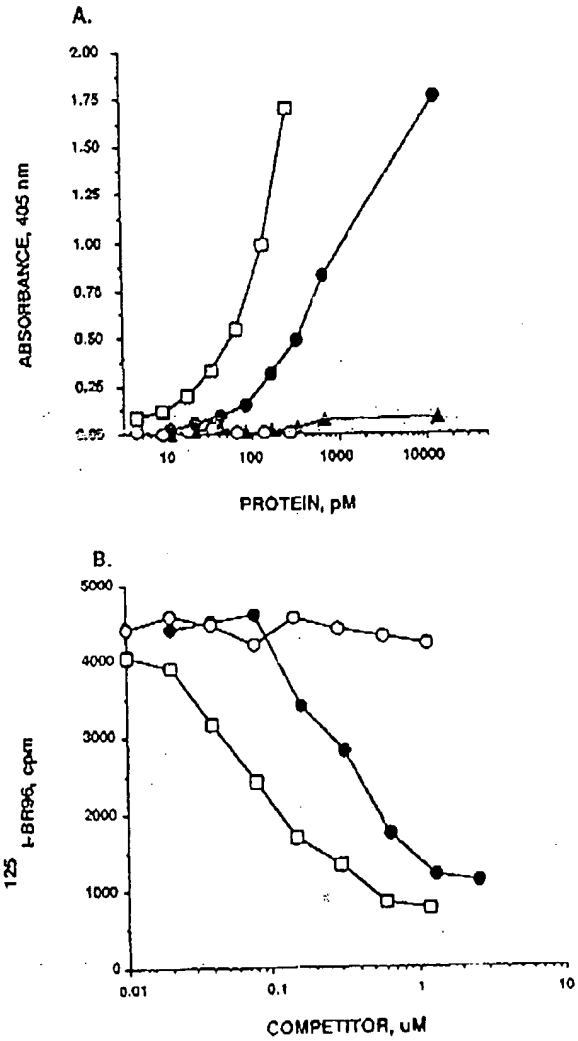

Fig. 3. Binding analysis of BR96 sFv-PE40. *A*, direct-binding analysis on ELISA plates coated with Lewis^y antigen and probed with BR96 anti-idiotypic antibody. A comparison of BR96 IgG (□), BR96 sFv-PE40 monomers (◆), BR96 sFv-PE40 aggregates (▲), and L6 IgG (○) is shown. *B*, competition of ^{125}I-labeled BR96 IgG with BR96 sFv-PE40 (●), BR96 IgG (□), and L6 IgG (○).

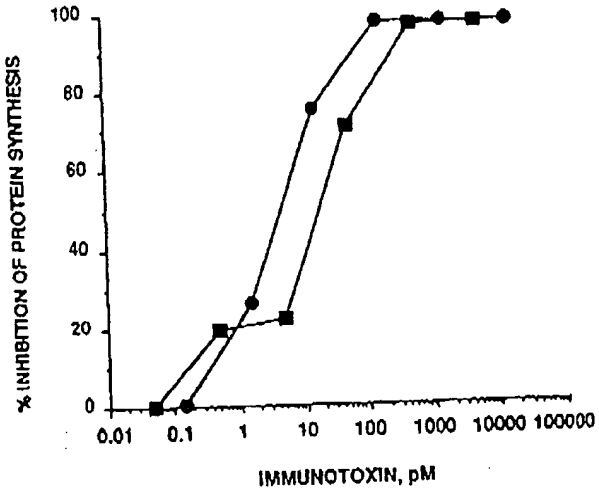

Fig. 4. Cytotoxicity analysis of BR96 sFv-PE40. Inhibition of protein synthesis in MCF-7 cells was determined for BR96 sFv-PE40 (●) and chiBR96-LysPE40 (■).

Table 1 *Cytotoxicity of BR96 sFv-PE40 on various cell lines*

ID_{50} is the amount of BR96 sFv-PE40 required to inhibit 50% of protein synthesis as determined by [^{3}H]leucine incorporation. After a 20-h incubation with the toxin the cells were pulsed for 4 h with [^{3}H]leucine. BR96 fluorescence intensity, specific BR96 fluorescence intensity minus nonspecific human IgG fluorescence.

Cell line	Cancer type	BR96 fluorescence intensity	IC_{50} ng/ml	IC_{50} pM
MCF-7	Breast	177.8	0.3	5
L2987	Lung	172.8	5.0	75
RCA	Colon	138.5	8.0	119
A2780	Ovarian	103.2	50.0	750
KB	Epidermoid	33.6	500.0	7462

unexpected. Mixed disulfides hinder binding activity, mainly because the antigen-combining sites are not correctly displayed. Perhaps, a truly bivalent recombinant molecule would bind as well as an IgG. Therefore, we have initiated experiments to produce bivalent BR96 sFv-PE40 for the purpose of increasing the antigen-binding activity of the recombinant immunotoxin molecule.

The BR96 antibody has been shown to identify a tumor-associated antigen expressed on carcinomas of the lung, colon, breast, and ovary (23). In a FACS analysis of five different cell lines, we have determined the distribution of BR96 antigen and have been able to correlate the cytotoxic potential of BR96 sFv-PE40 with the relative number of antigen on the surface of the target cells. We have found that BR96 sFv-PE40 is extremely potent against cancer cells displaying the BR96 antigen, with MCF-7 cells being the most sensitive line examined.

BR96 is one of only a few antibodies that not only binds solid tumor antigens but also internalizes rapidly. Other examples include anti-transferrin receptor antibody (32) and the carbohydrate antigen-specific antibody, B3 (33). When PE40 immunotoxins are prepared, there is a requirement for internalization via a ligand or antibody because PE40 lacks that capability since domain I has been deleted. Replacement of domain I with a selective mode of delivery, *i.e.*, by internalization of the immunotoxin following antigen-specific binding, redirects the toxin to the antigen. Both BR96 and B3 internalize in monovalent as well as bivalent form, as evidenced by their functional activity as PE40 conjugates and fusion proteins (13, 20, 34).

BR96 sFv-PE40 is more potent than the IgG chemical conjugate against the tumor cell lines tested in this study. Thus, the recombinant immunotoxin may prove to be a more powerful inhibitor of tumors than the chemical conjugate. To begin to address the potential antitu-

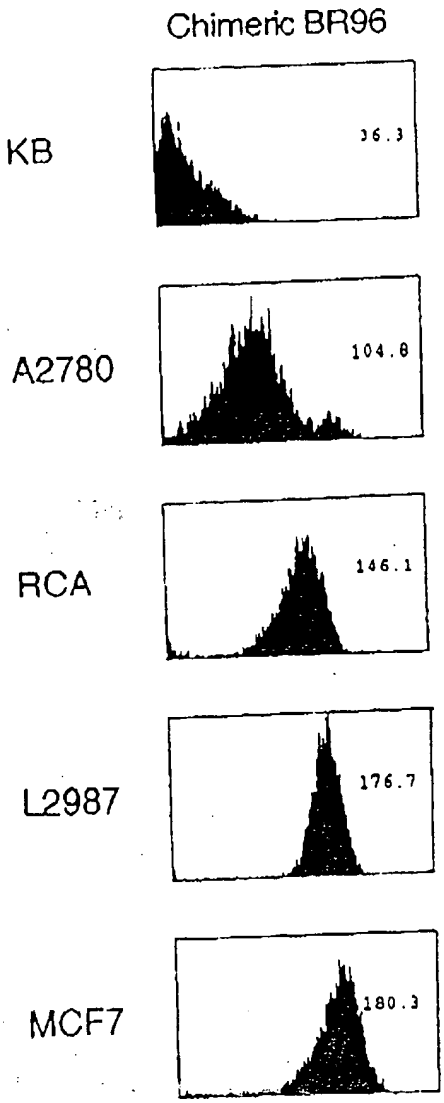

Fig. 5. FACS analysis of five human carcinoma lines. Data are displayed in each histogram as the mean channel number for BR96 IgG. The mean fluorescence for a human IgG control antibody for the cell lines was 2.7 (KB), 1.6 (A2780), 7.6 (RCA), 3.9 (L2987), and 2.5 (MCF-7). Fluorescence intensity for each cell line was determined by subtracting the human IgG mean channel number from the BR96 mean channel number.

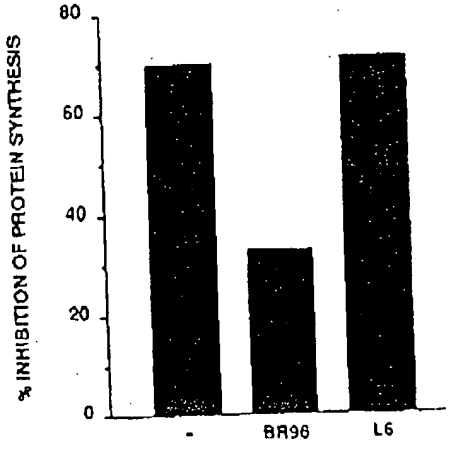

Fig. 6. Competitive cytotoxic analysis of BR96 sFv-PE40. Inhibition of protein synthesis in L2987 cells by BR96 sFv-PE40 (50 ng/ml) alone or in the presence of either BR96 IgG or L6 IgG (100 μg/ml).

Table 2 *Single-chain immunotoxin versus chemical conjugate immunotoxin. comparative blood level analysis*

^{125}I-BR96 sFv-PE40 and ^{125}I-chiBR96-LysPE40 were injected into athymic mice. The mice (2–4/group) were sacrificed at the indicated times. The % ID/ml for the blood was calculated as described in "Materials and Methods."

Time	BR96 sFv-PE40 (% ID/ml blood)	chiBR96-LysPE40 (% ID/ml blood)
5 min	49.8	57.5
15 min	43.9	54.8
30 min	28.2	46.3
60 min	15.5	41.5
2 h	8.6	23.4
4 h	5.2	22.0
6 h	2.5	20.5
24 h	0.2	7.2
48 h	0.1	3.6

mor activity of BR96 sFv-PE40, the chimeric toxin was administered i.v. to mice and found to have a $t_{1/2}\alpha$ of 28.5 min, as compared with that of the chemical conjugate chiBR96-LysPE40, which was 54 min. This is a noteworthy difference between these two molecules, because it may be an advantage for the single-chain immunotoxin that it is cleared more rapidly from the blood. The immunotoxin molecules are stable and retain biological activity following administration into animals.

This study shows the *in vitro* efficacy of the single-chain immunotoxin, BR96 sFv-PE40. Brinkmann *et al.* (13) reported on B3 sFv-PE38KDEL, a similar immunotoxin targeted to carcinomas that has antitumor activity against human tumor xenografts in athymic mice. Since BR96 has a different antigen specificity, BR96 sFv-PE40 is a novel fusion protein. Our future plans include studies with BR96 sFv-PE40 in athymic mice carrying tumor xenografts in order to further characterize this molecule as a potential agent for the treatment of cancer.

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This might explain the problems so far encountered in the studies of binding labelled peptides to MHC on intact cells[3,8]. At the concentration of peptide 17-29 (100 ng ml^{-1}) that we have routinely used, the ratio of binding to DR1 versus the other binding is favourable. The data summarized in Fig. 3*a* also indicate that the binding of peptide 17-29 to EHM cells approaches saturation at a level of class II-dependent binding of about 7×10^4 molecules per cell; at this level ~1% of the peptide offered is bound. This binding involves at most 15% of all DR molecules that are found on the membrane of an EBV-transformed B-cell line[17], which is in agreement with the fact that the majority of class II MHC binding sites are occupied by endogenous peptides[18].

Figure 3*b* shows the kinetics of binding of peptide 17-29 to EHM cells; maximum binding is achieved in 45 min, which is much shorter than the 6 hours or longer needed for maximum binding of peptides to purified class II MHC molecules[2,3]. In fact, in the same system of peptide 17-29 and DR1, the binding to isolated DR1 molecules is not even complete in 6 hours (T. Jardetsky, personal communication). It would therefore seem that the rapid binding to DR1 of influenza matrix peptide 17-29 seen with living cells is related to their biological activity and is not simply the consequence of the intrinsic high affinity of the system. Roosneck *et al.*[9] have seen that living B cells become fully competent to present a tetanus toxoid peptide to T cells after 40 minutes of exposure to it. Support for the concept that some activity of the living cells is necessary for the rapid loading of class II MHC with a peptide comes from experiments with glutaraldehyde-fixed EHM cells (Fig. 4), showing that these cells have lost the capacity for rapid binding of matrix peptide 17-29.

Fixation does not eliminate the class II MHC-dependent binding to the same cells when these are exposed to the peptide for 18 hours. This is in agreement with the previous report of Shimonkevitz *et al.*[7] showing that glutaraldehyde-fixed cells can stimulate T cells in a specific way when incubated in the presence of a peptide for 24 hours. It seems that the peptide-binding properties of class II MHC molecules in fixed cells are similar to those of the same molecules after purification. Figure 4 also shows that peptide binding by intact cells does not occur in the cold.

In conclusion, it seems that living B cells have a system for relatively rapid loading of class II MHC molecules with peptides. This system is probably not only operative at the level of the cell membrane, but also in intracellular vesicles where peptides derived from processing of internalized protein antigens are present. In this case, the MHC loading time must be shorter than the intracellular transit time of either the newly synthesized or the recycling class II MHC molecules. The molecular associations and structural events that make the rapid MH: peptide associations in living B cells possible remain to investigated.

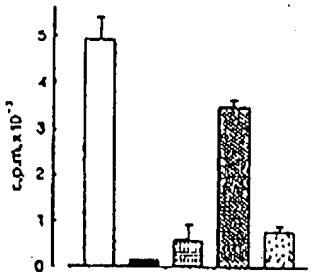

FIG. 4 Effect of glutaraldehyde fixation on binding of influenza matrix peptide 17-29-Tyr^{1,29}I. Cells were fixed with glutaraldehyde (Serva, Heidelberg) exactly as indicated by Shimonkevitz *et al.*[7]. They were then incubated for indicated periods of time at 37 °C with 10 ng peptide (in 0.1 ml medium with 10^6 cells). ▨ EHM cells, incubation 30 min; ▩ EHM cells incubation 18 h; flecked bar, MOLT 4 cells, incubation 18 h. In addition, the figure gives the data for unfixed EHM cells incubated for 30 min at 37 °C (open bar) or at 4 °C (filled bar). Every column gives the mean of triplicate cultures ± 1 s.d.

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A recombinant immunotoxin consisting of two antibody variable domains fused to *Pseudomonas* exotoxin

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ANTIBODIES and growth factors have been chemically couple to different toxins to produce cytotoxic molecules that selectivel kill cells bearing appropriate antigens or receptors[1,2]. Antibody toxin conjugates (Immunotoxins) produced using conventiona chemical coupling techniques have several undesirable characteris tics. The smallest binding unit of an antibody is an Fv fragmen which consists of a light and heavy chain variable domain. Recently active single chain Fv fragments of antibodies have been produce in *Escherichia coli* by attaching the light and heavy chain variabl domains together with a peptide linker[3,4]. Here we describe th construction and expression in *E. coli* of a single chain antibod toxin fusion protein, anti-Tac(Fv)–PE40, in which the variabl regions of anti-Tac, a monoclonal antibody to the p55 subunit o the human interleukin-2 receptor[5], are joined in peptide linkag to PE40, a modified form of *Pseudomonas* exotoxin lacking it binding domain. Anti-Tac(Fv)–PE40 was very cytotoxic to tw interleukin-2 receptor-bearing human cell lines but was no cytotoxic to receptor-negative cells.

Immunotoxins made by chemically attaching a toxin to a intact antibody contain the constant region of the antibody which is not necessary for immunotoxin action, but which reduces its access to target cells outside the circulation, and increases its immunogenicity. Furthermore, the product i

heterogeneous and the yields are often poor. Recombinant DNA techniques have been used to produce chimaeric growth factor–toxin fusion proteins in *E. coli*[6–10]. These molecules can be readily purified in large amounts and contain only the sequences required for specific cell recognition and the cytotoxic activity of the toxin. Because the antigen-binding site of an antibody is composed of two separate polypeptide chains, it has been difficult to produce antibody-toxin chimaeric proteins in *E. coli*. To create a single-chain recombinant immunotoxin, we assembled a plasmid, pVC70108, which contains a 348-base pair (bp) DNA segment encoding an anti-Tac heavy chain variable domain (VH) joined to a 318-bp DNA segment encoding a light chain variable domain (VL) by a 45-bp linker; VL was in turn joined to a DNA segment encoding amino acids 253–613 of *Pseudomonas* exotoxin (PE) (Fig. 1). (The cloning and sequence of the anti-Tac variable regions will be described elsewhere.) The assembled gene is under the control of a T7 promoter[11]. The authenticity of the coding region of the plasmid was confirmed by DNA sequencing (data not shown). Upon induction with isopropyl-β-D-thiogalactoside (IPTG), BL21 (λDE3) carrying plasmid pVC70108 produced large amounts of a protein of relative molecular mass (M_r) ~65,000 (65 K), as shown by SDS-PAGE (lane 1, Fig. 2*b*). On immunoblots, the 65 K chimaeric protein reacted with an antibody to PE (data not shown). The fusion protein was mostly contained in the 100,000*g* pellet (lane 3, Fig. 2*b*) of the sonicated spheroplasts (lane 2, Fig. 2*b*). This pellet was used as the source to prepare anti-Tac(Fv)-PE40. Guanidine hydrochloride denaturation followed by rapid dilution was used to solubilize and renature the chimaeric protein[8]. The renatured protein was applied to a Mono Q column and the monomeric form of the fusion protein eluted at 0.2–0.22 M NaCl (Fig. 2*b*, lane 4 and Fig. 2*a*). High-molecular weight aggregates were eluted at higher ionic strength (fractions 42–50; Fig. 2*a*). Further purification of the chimaeric protein was carried out on a TSK-250 gel filtration column; the chimaeric protein eluted as a symmetrical peak at the location expected for a 65 K protein (data not shown). SDS-PAGE showed the protein to be >95% pure (lane 5, Fig. 2*b*) and N-terminal amino acid analysis showed the protein had the expected sequence, Met-Gln-Val-. Highly purified monomeric anti-Tac(Fv)-PE40 (~200 μg) was obtained from 1 litre of cells grown to an optical density at 650 nm of 0.6 before induction.

The anti-Tac antibody binds to the p55 subunit (Tac antigen, low affinity receptor) of the interleukin-2 (IL-2) receptor, which is present in large amounts on HUT-102 cells[5]. Therefore, the chimaeric protein was initially tested for cytotoxicity on HUT-102 cells. Anti-Tac(Fv)-PE40 inhibited protein synthesis in a dose-dependent manner with a 50% inhibitory dose (ID_{50}) of 0.15 ng ml^{-1} (2.3×10^{-12} M) in a 20 h assay (Fig. 3; Table 1). At concentrations >4 ng ml^{-1}, there was complete inhibition of protein synthesis. Several specificity controls were carried out. Addition of excess anti-Tac (10 μg ml^{-1}) prevented the cytotoxicity of anti-Tac(Fv)-PE40 on HUT-102 cells, whereas a control monoclonal antibody, OVB3, directed against an antigen found on ovarian cancer cells[12] did not (Fig. 3). The human T-cell leukaemia line Cr H.2 (ref. 13), which has a lower numbe

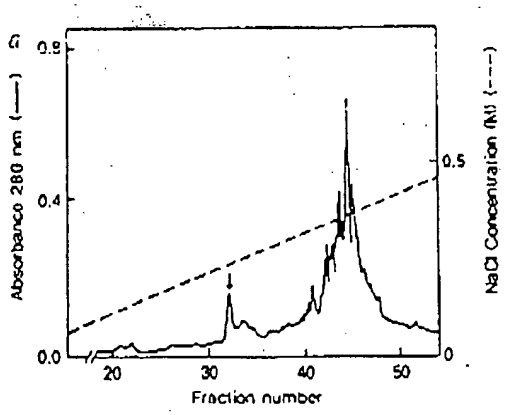

FIG. 2 Purification and characterization of anti-Tac(Fv)–PE40. *a*, Mono Q column chromatography of renatured soluble anti-Tac(Fv)–PE40. Renatured material was applied on a Mono Q column; proteins were eluted with a NaCl gradient (0–0.5M) and 4 ml fractions were collected. The position of active monomeric anti-Tac(Fv)–PE40 is shown by a vertical arrow. *b*, SDS-PAGE of samples at various stages of purification. The gel was stained with Coomassie blue. Lane 1, total cell pellet; lane 2, spheroplasts; lane 3, 100,00 *g* pellet of sonicated spheroplasts; lane 4, pool of fractions (32–33) from the mono Q column; lane 5, pool of peak fractions from the TSK-250 column; lane 6, native PE, M_r 66 K. Molecular weight markers (K) are indicated.
METHODS. *E. coli* strain BL21 (λDE3) carrying plasmid pVC70108 was grown, induced with IPTG, and the cell pellet was processed as described previously[6–8]. Dithiothreitol was omitted from denaturation buffer and renaturation was carried out for 16 h. After renaturation and dialysis, the sample was applied on a Mono Q column (HR 10/10) at 3 ml min^{-1}. The column was washed with 40 ml Buffer A (Tris-HCl 20 mM, pH 7.6) and developed with a 200 ml linear gradient (0–0.5 M NaCl). Eluted proteins were monitored at 280 nm. Fractions (4 ml) were collected and tested for cytotoxicity on HUT-102 cells. For SDS-PAGE, samples were boiled with Laemmli sample buffer[14] and electrophoresed on a 10% gel.

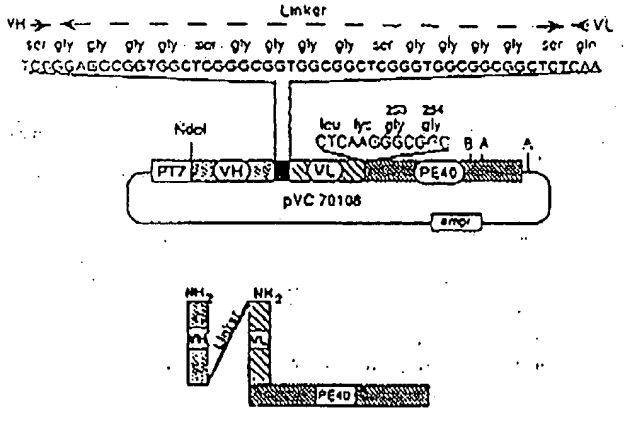

FIG. 1 a, Expression plasmid for anti-Tac(Fv)–PE40. Expression plasmid pVC70108 contains a fusion gene encoding various domains of anti-Tac (the variable domain of the heavy chain (VH, first 116 amino acids of mature heavy chain), a 15-amino-acid linker ((Gly-Gly-Gly-Gly-Ser)$_3$), and the variable domain of the light chain (VL, first 106 amino acids of mature light chain)) and amino acids 253–613 of PE (refs 6–9, 17) as a single polypeptide chain. The gene is under control of a T7 promoter linked to a Shine-Dalgarno sequence and initiation codon (PT7) as described previously[6–8,17]. *E. coli* strain BL21 (λDE3) carrying pVC70108 was used to express the chimaeric protein upon IPTG induction. Amp^r, β lactamase gene; B, *Bam*HI; A, *Ava*I. *b*, Schematic arrangement of various domains of anti-Tac(Fv)–PE40. The hybrid protein is shown as a single polypeptide chain. The C-terminus of VH is joined to the N-terminus of VL through a 15 amino-acid linker as shown in *a*. Details of the construction will be described elsewhere or can be obtained from the authors.

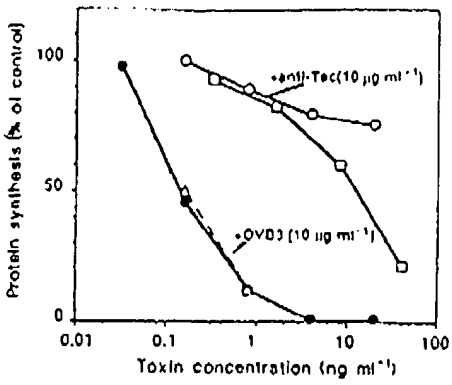

FIG. 3 Cytotoxicity of anti-Tac(Fv)–PE40 and anti-Tac-PE40 on HUT-102 cells expressing IL-2 receptors. Cytotoxicity was determined by measuring protein synthesis in HUT-102 cells after treatment with: (●) anti-Tac(Fv)–PE40; (○) anti-Tac(Fv)–PE40 + 10 μg anti-Tac; (△) anti-Tac(Fv)–PE40 + 10 μg OVB3; (□) anti-Tac–PE40.
METHODS. HUT-102 cells were washed twice with serum-free medium and plated in RPMI 1640 medium with 5% fetal bovine serum at 3×10^5 cells per well in 24-well plates. Various dilutions of recombinant anti-Tac(Fv)–PE40 and chemically conjugated anti-Tac–PE40 were prepared in PBS with 0.2% human serum albumin and added to appropriate wells. After 20 h the cells were labelled with [^{3}H]leucine for 90 min and the radioactivity in the TCA precipitate of the cell pellet determined. The results are expressed as % of control with no toxin added. For competition, 10 μg of anti-Tac or OVB3 were added to each well just before adding anti-Tac(Fv)–PE40.

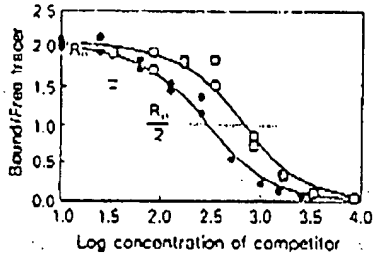

FIG. 4 Competition binding analysis of anti-Tac versus anti-Tac(Fv)–PE40. Competition of anti-Tac(Fv)–PE40 (open squares) and native mouse anti-Tac (solid circles) with [^{125}I] labelled tracer anti-Tac to bind to Tac antigen on HUT-102 cells is shown. Solid lines are computer generated idealized curves that model binding competition. Ro is the bound/free ratio for tracer in the absence of competitor, and Ro/2 is the 50% inhibition point for tracer binding, from which a binding affinity of $3.5 \times 10^9\ M^{-1}$ for anti-Tac(Fv)–PE40 is calculated, compared with $9.7 \times 10^9\ M^{-1}$ for native anti-Tac.
METHODS. [^{125}I]labelled anti-Tac (2 μCi μg^{-1}) as tracer was used at 1.5 ng per assay with varying concentrations of competitor and 4×10^5 HUT-102 cells as source of Tac antigen in 0.2 ml of binding buffer (RPMI 1640 with 10% fetal bovine serum, 100 μg ml^{-1} human IgG, 0.1% sodium azide), and incubated at room temperature with mixing for 2 h. Under these conditions, the concentration of tracer is 50 pM and Tac peptide 500 pM. Free tracer is 10 pM by calculation and satisfies the condition that free tracer be less than $1/K_a = 100$ pM (using $10^{10}\ M^{-1}$ for anti-Tac K_a) for the assumptions of the competition analysis[19]. Assays were performed in parallel with a control cold anti-Tac antibody, and curve shifts at the 50% inhibition point of bound/free tracer binding (Ro/2) versus log competitor concentration were quantitated. The concentrations were obtained from the antilogs of the abscissa, and the affinity constant K_a for the construct, X, derived from the formula[19] $([X]_{1/2} - [\text{anti-Tac}]_{1/2}) = 1/K_a \sim 1/K_a$ where $[\ ]_{1/2}$ indicates the concentration of competitor at which tracer binding is Ro/2. Standard Scatchard plotting of binding data with anti-Tac gave linear graphics and a K_a of $9.7 \times 10^9\ M^{-1}$, comparable to that obtained by other investigators[20]. The K_a of $3.5 \times 10^9\ M^{-1}$ for anti-Tac(Fv)–PE40 was calculated from the above formula. All concentrations were measured by Bradford protein microassay against a standard curve with human IgG (ref. 20). For competition analysis, these concentrations were normalized on the basis of the bindable fraction obtained in separate tests with radiolabelled anti-Tac(Fv)–PE40 (0.44) and radiolabelled anti-Tac (0.8) with excess HUT-102 cells[16] to yield concentrations of bindable protein for the abscissa.

TABLE 1 Cytotoxicity of anti-Tac(Fv)–PE40 on various cell lines

Cell line	IL-2 receptors per cell: Low affinity	IL-2 receptors per cell: High affinity	ID_{50} (ng ml^{-1}, 24 h)
HUT-102	94,000	3,800	0.15
Cr II.2	12,000	350	2.7
CEM	<20	<20	>1,000
OVCAR-3	—	—	>1,000
KB	—	—	>1,000
A431	—	—	>1,000

Cell lines OVCAR3, KB and A431 were seeded at 1×10^5 ml^{-1} in 24-well plates one day before the addition of toxin. HUT-102, Cr II.2 and CEM were washed twice and seeded at 3×10^5 ml^{-1} in 24-well plates (see also Fig. 3). RPMI 1640 with 10% fetal bovine serum was used for Cr II.2. Various dilutions of toxin preparations were added, and 20 h later the cells were labelled for 90 min with [^{3}H]leucine. The radioactivity in the trichloroacetic acid precipitate of the cells was determined. ID_{50} is the concentration of toxin that inhibits protein synthesis by 50% as compared with a control with no toxin added. All the assays were done in duplicate and repeated three times. (Data for the number of low and high-affinity IL-2 receptors on the various cell lines is from the unpublished data of T. Waldmann and ref. 16.)

of both low- and high-affinity IL-2 receptors than HUT-102, was also sensitive to anti-Tac(Fv)–PE40, with an ID_{50} of 2.7 ng ml^{-1} (Table 1). Furthermore, several human cell lines without IL-2 receptors, including the T-cell leukaemia line CEM, as well as carcinoma cell lines A431, KB and OVCAR-3 (ref. 14), were not affected by anti-Tac(Fv)–PE40, even at 1 μg ml^{-1} (Table 1).

Previously we reported that anti-Tac chemically conjugated to PE- or PE40-killed HUT-102 cells[14,15]. When thioether conjugates are made, anti-Tac-PE had an ID_{50} of 1.2 ng ml^{-1}, and anti-Tac–PE40 similarly prepared had an ID_{50} of 13 ng ml^{-1}. As anti-Tac(Fv)–PE40 (65 K) is about 30% smaller by weight than anti-Tac–PE (216 K), the chimaeric toxin is on a molar basis several times more active than anti-Tac–PE and considerably more active than anti-Tac–PE40. Anti-Tac–PE is a heterogeneous chemical conjugate in which the two molecules are connected by a thioether bond and different lysines in PE and anti-Tac are used in the conjugation reaction. In anti-Tac–PE40, the attachment appears to be mainly through the lysine residues in domain III of PE40 and this reduces the activity of the PE conjugates[14]. IL-2–PE40 is another chimaeric molecule which was constructed by fusing a complementary DNA for human IL-2 to PE40 sequences[7]. IL-2–PE40 is slightly less cytotoxic to HUT-102 cells than anti-Tac(Fv)–PE40, with an ID_{50} of 1–5 ng ml^{-1}.

Competition binding studies showed an affinity of $3.5 \times 10^9\ M^{-1}$ for anti-Tac(Fv)–PE40, ~3-fold lower than that of anti-Tac, measured at $9.7 \times 10^9\ M^{-1}$ (Fig. 4). This can be compared with a fourfold loss of affinity of a Fv construct versus Fab fragment of anti-bovine growth hormone[3] and a sixfold loss for the Fv construct versus an Fab fragment of anti-digoxin[4]. The affinity of Fv binding for p55 seems to be preserved to a greater extent than similar preparations of anti-bovine growth hormone and anti-digoxin antibodies.

In summary, we have created an active immunotoxin in *E. coli* by the fusion of cDNAs encoding the anti-Tac variable regions with a fragment of DNA encoding a modified form of *Pseudomonas* exotoxin. Using this approach it should now be possible to create active recombinant immunotoxins with other antibodies.

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Vesicular transport between the endoplasmic reticulum and the Golgi stack requires the NEM-sensitive fusion protein

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AN *N*-ethylmaleimide-sensitive fusion protein (NSF) has been purified on the basis of its ability to catalyse vesicular transport within the Golgi stack. We report here that this same protein is required for transport from the endoplasmic reticulum to the Golgi stack in semi-intact cells. This transport process is inhibited by a monoclonal antibody against NSF. Furthermore, pretreatment of semi-intact cells with *N*-ethylmaleimide, a sulphydryl alkylating reagent, inhibits transport. Addition of highly purified NSF largely restores transport from endoplasmic reticulum to Golgi. These results suggest that NSF is a general component of the transport machinery required for membrane fusion at multiple stages of the secretory pathway.

The *SEC18* gene of yeast encodes an NSF activity that will function in place of animal cell NSF with animal cell Golgi membranes[1]. As mutants in the *SEC18* gene are defective in transport from the endoplasmic reticulum (ER) to the Golgi in yeast[2], we wondered whether NSF is also required for this transport step in animal cells, in addition to its established role in promoting fusion within the confines of the Golgi stack. To address this issue we have examined whether NSF is needed for transport of the vesicular stomatitis virus (VSV)-encoded glycoprotein (G protein) between the ER and *cis* Golgi compartment in an *in vitro* system[3] in which semi-intact cells prepared from VSV-infected Chinese hamster ovary (CHO) cells are incubated in the presence of cytosol and ATP.

A complete incubation mix containing semi-intact cells, cytosol and ATP was treated with *N*-ethylmaleimide (NEM) on ice for 15 min (conditions which inactivate NSF[4]) before incubation. Subsequent transport (during an incubation at 30 °C for 90 min) was inhibited by more than 90 per cent (Fig. 1, lane iv). To determine whether the NEM-sensitive factor required for delivery of VSV-G protein from the ER to the *cis* Golgi compartment could be replaced by NSF, NEM-treated semi-intact cells and NEM-treated cytosol were incubated together at 30 °C in the presence or absence of NSF purified from CHO cells as described[5]. Addition of NSF stimulated transport about 10-fold over background (Fig. 1, compare lanes iv and v) restoring activity to a level which was nearly 50 per cent of that of an untreated incubation that had been supplemented with NSF (Fig. 1, lane iii). In this instance, NSF only marginally (10%) stimulated an incubation that had not been pretreated with NEM (Fig. 1, compare lanes ii and iii). But some preparations of semi-intact cells (apparently more deficient in NSF) are stimulated by up to 30 per cent (see Fig. 2). These results strongly

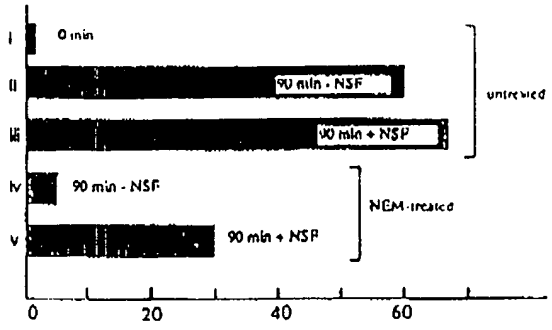

FIG. 1 NSF is required for transport from ER to Golgi. Assay conditions were as previously described[3,6]. Lanes i–iii, semi-intact cells, cytosol and ATP were incubated at 30 °C for the indicated time in the absence (lane ii) or presence (lane iii) of 0.16 μg of NSF (purified from CHO cells as described[5] in a final volume of 40 μl. Lanes iv and v, semi-intact cells and cytosol were pretreated with 1 mM NEM for 15 min at 0 °C. Subsequently, glutathione was added to a final concentration of 2 mM to quench unreacted NEM. NEM-treated membranes and cytosol were incubated in the presence of ATP at 30 °C for 90 min in the absence (lane iv) or presence (lane v) of 0.16 μg NSF. The fraction of total VSV-G transported was measured as the fraction of G protein in the Man_5 form as described previously[3,6].

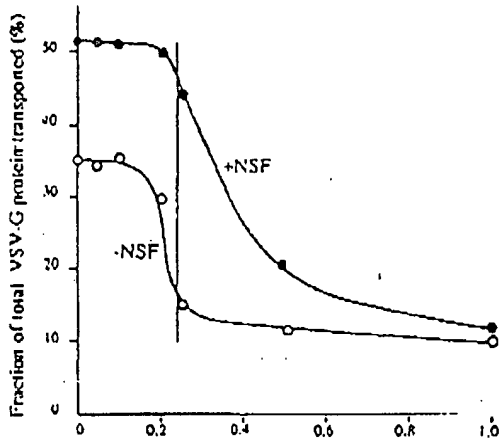

FIG. 2 Anti-NSF antibody inhibits the transport of VSV-G protein to the *cis* Golgi compartment. Assay conditions were as previously described[3,6]. The indicated amount of anti-NSF antibody was added to a 40 μl complete cocktail containing semi-intact cells, cytosol and ATP for 15 min on ice. Subsequently, the cocktail was transferred to 30 °C and incubated for 90 min in the absence (open circles) or presence of 0.16 μg purified NSF. The anti-NSF antibody (4A6) is an IgM whose properties have been described previously[6], and was purified from ascites fluid as described[3].

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to $CH_3.H^*COH.COOH$ (the L-lactic acid isomer) but has been rationally engineered (Fig. P56) to specifically transform substrates with very large side chains such as $-CH_2.CH.(CH_3)_2$ as required for modern single-compound drug production (*see* BIOTRANSFORMATION and BIODEGRADATION).

Engineering new catalytic properties into enzymes is still rare as the protein designer must construct the new substrate-binding site without altering the precise arrangement in space of the catalytic residues. A less rational but simpler approach to engineering new enzymes is to raise an antibody to a transition state analogue of the substrate. Any device which stabilizes the transition state of a chemical reaction will catalyse that reaction (*see* MECHANISMS OF ENZYME CATALYSIS). Such antibodies therefore often have weak catalytic activity; they are named ABZYMES (*Antibody-enzymes*). The weak activity can be improved by using protein engineering to insert extra catalytic amino acids into the antibody molecule.

A unique achievement is an abzyme catalysing the Diels–Alder chemical reaction. No natural enzyme is known to catalyse this carbon–carbon bond-forming reaction.

Engineered antibodies

Protein engineering has been exploited in the design of engineered antibodies [2] for medical use in humans. Highly specific MONOCLONAL ANTIBODIES have many applications in medicine. But most have to be made via the mouse HYBRIDOMA route; and the human body tends to react against these mouse (or other animal) antibodies, neutralizing their activity and clearing them rapidly from the body. 'Humanized' antibodies, which escape neutralization by the human immune system, can be produced by protein engineering. DNA encoding the specific antigen-binding site on the mouse antibody can be inserted by genetic engineering into the 'framework' DNA derived from a suitable human antibody gene.

This engineered gene can then be inserted into an appropriate cultured cell to produce the mouse–human hybrid antibody. As most of the surface amino-acid residues of the engineered protein are derived from a human antibody the immune system does not neutralize it. The recently developed ability to make in bacteria large quantities of antibodies from a synthetic gene that codes for a 20-residue peptide which links the heavy and light chain COMPLEMENTARITY DETERMINING REGIONS (CDRs) now avoids the expense and limitation of antibody production in cultured animal cells. For example, engineered hybrid antitumour antibodies that regress tumours by selectively binding to malignant but not normal cells have been produced in this way.

J.J. HOLBROOK

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protein evolution *See*: MOLECULAR EVOLUTION: SEQUENCES AND STRUCTURES.

protein export *See*: BACTERIAL PROTEIN EXPORT; PROTEIN SECRETION.

protein fingerprint A pattern of spots, characteristic of the protein concerned, which is produced by visualization of fragments separated by two-dimensional ELECTROPHORESIS of a digest of the purified protein by a specific cleavage reagent such as the proteinase trypsin.

Protein folding

THE biological activity of a protein depends on its folding into a highly organized three-dimensional structure under physiological conditions. The central questions of protein folding are:

1 Why does it occur? What are the forces that make a well-defined conformation thermodynamically favourable?

2 How does it occur? What mechanisms permit rapid and efficient self-organization?

3 How is the 3-D structure encoded by the amino-acid sequence?

These issues are, of course, intimately related and the goal of applying the principles of folding to, for example, predict structures from sequence or to design sequences compatible with particular structures (and functions?) (*see* PROTEIN ENGINEERING) is likely to require a broad understanding of each. At present there is still some way to go towards this objective but folding is the subject of intensive study.

Although the folding of all proteins must, of course, obey the same physicochemical laws, the balance of forces involved is likely to be very different in determining the structures of, for example, water-soluble proteins compared with those that traverse membranes. To date the vast majority of studies have focused on globular proteins in aqueous solution and these are considered in this discussion.

The native state

The conformation of a protein under conditions where it is biologically active is called its native structure (*see* PROTEIN STRUCTURE). Such structures can be determined in detail by X-RAY CRYSTALLOGRAPHY and NMR SPECTROSCOPY. Globular proteins are organized into compact structural DOMAINS, larger proteins comprising several such domains with varying degrees of interaction between them. Although these domains may depend on their mutual interaction for stability, they can be regarded as quasi-independent folding units. There are obviously wide differences in the details of the 3-D structures of different proteins but it now seems that there may be only a limited number of gross chain topologies compatible with forming a stable structural domain. It is quite commonly observed that proteins with no obvious sequence homology fold into similar structures; for example, the IMMUNOGLOBULIN FOLD is not only found in a huge range of related proteins in the IMMUNOGLOBULIN SUPERFAMILY